Aalto University
School of Chemical
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School of Chemical Technology
Degree Programme of Materials Science and Engineering

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Surface Enhanced Raman Spectroscopy detection of trace chemicals in inhomogeneous fluid mixtures

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ABSTRACT OF THE
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Detecting trace chemicals in heterogeneous fluid mixtures has a variety of possible applications for example in food industry. The drivers of this development have not only been build on consumer awareness but have also been emphasized by decision makers at the EU level. The current laboratory methods are laborious, expensive and slow, which challenges their application in milk quality control especially in terms of qualitatively detecting such chemical residues an melamine. More studied are therefore needed of methods that are applicable as field devices for consumer use and as on-line analysers for industrial contexts.

The more complex the media the more challenging the detection of a chemical. Separating relevant information from the raw measurement data poses a difficult task. Moreover the sample preparation should be designed to guarantee stable measurement performance for obtaining usable data. In this thesis work the sample preparation chain and substrate handling are looked into. The issues most severely hindering the detection are stem from sample preparation and Surface Enhanced Raman Spectroscopy substrate performance. Which constitute fundamental issues requiring further examination. Due to the high amount of uncontrolled variables, the results stated here are not statistically tested.

The project achieved to produce sample preparation schemes that result in samples of different types of spreading on the substrates. Furthermore different kind of noise noise was seen in the spectra of the two sample preparation schemes. The development in data treatment and possible further investigation of the sampling chain together dictate the direction in which sample pretreatment ought to be taken.

Keywords: SERS, sampling, sample pretreatment, inhomogeneous mixtures, trace chemicals, melamine, milk

AALTO-YLIOPISTO
KEMIAN TEKNIIKAN KORKEAKOULU

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Kemikaalijäämien mittaaminen on olennainen osa monia prosessiketjuja. Alati kasvavaa tarvetta pienten pitoisuuksien määrittämiselle hyvinkin heterogeenisissta näytteistä ajavat monet tekijät. Näistä merkittävin lienee tervetyttä ja turvallisuutta koskeva lainsäädäntö. Vaikka nykyiset laboratoriomenetelmät usein mahdollistavatkin tarkan määrityksen, analyysien hitaus, korkeat kustannukset ja vaadittu korkeasti koulutetun henkilökunta rajoittavat menetelmien käytön laboratoroihin.

Tässä työssä pyrittiin kehittämään näytteen esikäsittelyä kuluttajakäyttöiseen SERS-analyysimenetelmään melamiinin pitoisuuden määrittämiseksi maidosta. Jotta SERS-anturi toimisi optimaalisesti maitonäyte pitää esikäsitellä, mutta suurimmat haasteet liittyvät edelleen kerätyn datan käsittelyyn, sekä menetelmän oikeellisuuden ja virheen arvioimiseen.

Avainsanat: SERS, näytteenotto, heterogeeninen näyte, näytteen esikäsittely, kemikaalijäämät, melamini, maito

Preface

This M.Sc. thesis was prepared in the department of Micro and Nanotechnology at the Technical University of Denmark in the fulfilment of the requirements for acquiring a master degree in Materials Technology in Aalto University, Finland, within a Nordtek exchange program. Thanks to Professor Michael Gasik, Assistant Professor Professor Michael Stenbæk Schmidt and researcher Michael Bache for suggesting me such an interesting and challenging topic for my thesis. Furthermore, the open-minded and inspiring contribution from my supervisors created a patient and encouraging atmosphere to work and experience a bit of Denmark too.

The experiments were carried also out in the premises of Danchip and DTU CEN. Despite some occasional frustration with AOE, their services and training were invaluable and humor spiced backup form Conny was most appreciated. I am glad I got to experience the Nanoprobes way of working, the precious lessons and ideas to embrace, and take home with me. I am grateful for Aalto University and DTU Nanoprobes allowing this opportunity to be part of this cutting edge, awesome, research group. Additional thanks to Kasper Frhling, Anna Line Brgger, Tomas Rindzevicius, Julia Dyrnum and Anil Haraksingh Thilsted for valuable feedback and support during this final project.

Lastly but not least, to friends and family, a big thanks and a hug! Thank you for mum, dad, Wilhelmiina and Elisa for the support and caring for Biffe & Keke while I have been gone.

Otaniemi, 18.9.2015

Tiainen L. K. B.Sc. (Tech)

Contents

1 Introduction

The need for methods to detect trace chemicals in complex fluid mixtures has become increasingly important due to national and international requirements. A recent example of this need can be seen in the Chinese melamine milk and the American pet food scandals [U.S. Food & Drug Administration, 2007]. In both cases, foreign or inferior ingredients were introduced to these products during the production process in order to gain financial profits at the expense of consumer health. In the melamine milk scandal, melamine was used to fraud the protein content of milk-based products, thus allowing the producers to water down milk or to use lower quality ingredients. In China, over 50,000 children received treatment for urinary complications and infant fatalities were associated with melamine-spiked infant formula milk powders [Hau et al., 2009]. Melamine and its associates in milk, powdered milk and infant formulae can be quantitatively detected using electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS). However, this method as specified in the ISO standardISO/TS 15495 (IDF/RM 230:2010) is a laboratory technique that requires highly educated staff and expensive instrumentation. Moreover, obtaining results using this method is time-consuming due to the need to ship the samples to a laboratories.

The increasing demand for monitoring streams of chemicals has driven the development of more readily accessible, affordable and easy-to-use measurement systems. Technology is needed not only for consumer use but for industrial applications too. Moreover the levels of chemical compounds that we want to be able to measure are decreasing. Thus making it more difficult for conventional methods to meet that demand. Abundant molecules that may induce a hazard are to be monitored according to the EU Priority Substance Directive that sets the environmental quality standards. Perhaps one of the most efficient drivers in development of analyzing methods is the health & safety and environmental legislation. The EU Commission proposes "*... improvements to the monitoring and reporting of chemical pollutants in water, as well as a mechanism to obtain better information on the concentrations of other pollutants that might need to be controlled in the future at EU level.* [European Commission Press Release Database, 2012] Consequently, compelling industries to improve their knowledge over processes and increase control over product and waste quality.

Choosing a detection method for complex media is a process that optimizes several variables. Mostly the ruling ones are the accuracy of the measurement, the costs of implementation and operation, and the consequence of a false result. Additionally the quality of the data to be gathered sets a demand for the analysis - qualitative or quantitative. The question is if SERS is a future detection method for highly saturated, complex and common industrial product, such as milk.

This thesis focuses on sampling in complex media, which presents challenges in detecting a known chemical in a poorly known sample. In addition, the thesis assesses

issues arising from the use of poorly known samples and proposes some solutions. The objective was to design a milk sample preparation procedure that would yield a detection limit of 1 ppm of melamine in milk. The basis for the experimental work was to develop a field device sampling procedure that would be robust, user friendly and appropriate for qualitative detection.

The remainder of this thesis is divided into ten chapters. Chapter 2 presents the background for melamine detection and the described the SERS technology. Chapter 3 introduces the SERS substrate. Chapters 4 and 5 describe milk and preparation schemes to improve the melamine detection. Results are presented in Chapter 6 followed by proposals for analytical methods in Chapter 7. Chapter 8 focuses on the sampling chain optimization. Finally the findings are concluded, discussed and recommendations for future research are proposed.

2 Background

2.1 Surface enhanced Raman spectroscopy

Surface enhanced Raman scattering provides greatly enhanced Raman signal when a molecule is adsorbed on a rough metal surface or colloids that aggregate or at the sharp edges or tips of individual nano structures. The mechanism is not completely understood but is believed to be a combination of two processes an electromagnetic (EM) and a chemical enhancement.

Excitation wavelengths that are useful in Raman spectroscopy cover a region from NIR to UV. The Raman bands in the spectrum are not changing as different wavelengths used, but their intensities differ. Furthermore, the SERS spectra differ from conventional Raman spectrum. The selection of the wavelength influences the amount of fluorescence and information gained on chemistry. For example, aromatic and conjugated structures absorb UV light, so resonance from these structures is enhanced when UV light source is used for excitation.

In the chemical enhancement is thought to lead to an average enhancement factor of 100 through a charge transfer state that is created between the metal and adsorbate. This mechanism is site-specific and analyte-dependent [Haynes et al., 2005]. The greater impact is due to EM enhancement mechanism which contributes with an average enhancement factor of $\geq 10\,000$ and is a direct consequence of roughness of the metal surface.

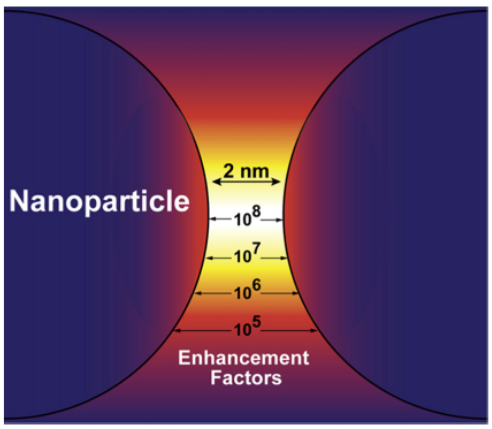


Figure 1: An illustration of a hotspot between NPs, SERS enhancement factor with respect to relative position. [Petryayeva and Krull, 2011]

The Raman scattering intensity is proportional to the square of the induced dipole moment as EM enhancement rises from incident electromagnetic field intensity and the polarizability. Chemical enhancement is due to interactions between the analyte and the metal surface. The enhancement is then a product of both enhancement mechanisms and dynamic process where molecules may move in and

out of the hot spot and change their orientation [Petryayeva and Krull, 2011]. The vast enhancement at the hot spots generated a concept of single molecule detection. Suitable conditions are proposed to exists at the junctions of compactly aggregated nanoparticles.[Haynes et al., 2005]

2.2 Nanopillars as SERS substrate

Freestanding nanopillars with silver or gold coating can be used as effective SERS substrates. Nanopillars may lean together as solution evaporates and capillary forces pull them together forming so hot spots that trap analyte molecules as illustrated in Figure 2 [Hu et al., 2010].

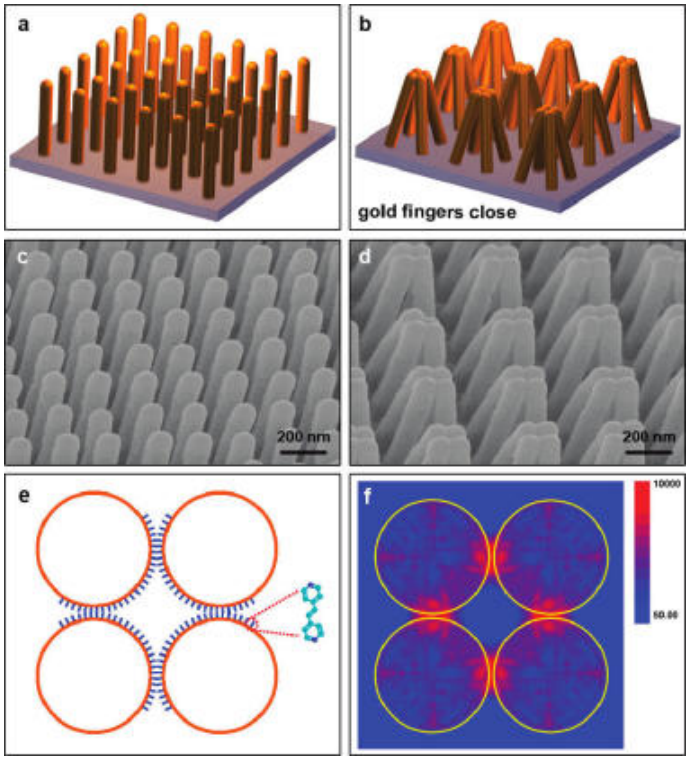


Figure 2: Goldnanofingers.(a,b) Schematic of capillary force driven closing mechanism. (c,d) SEM images of fingers before and after closing. (e) Schematic of BPE molecules trapped between the fingers. (f) Distribution of electric field intensity $|E(r)|^2$ at 750 nm for four Au spheres of 68 nm radius. [Hu et al., 2010]

The leaning of the pillars may not be an unconditional requirement for surface enhancement and non-leaning pillars can work as efficient SERS substrates too. [Discussions with, Rindzevicius T. and Thilsted A., May 2015, DTU, Denmark]

2.3 Melamine

Melamine (2,4,6-Triamino-1,3,5-triazine, sym-Triaminotriazine) is an organic base, pKa ≈ 8. The most favorable tautomeric forms of melamine are presented in the figure 3. The amide structure is the most stable in neutral pH. [Klotz and Askounis, 1947].

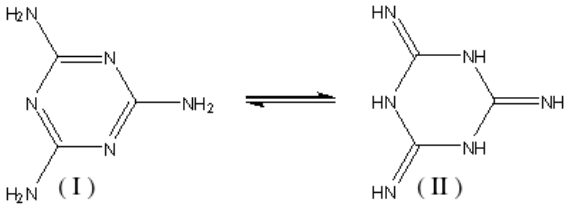


Figure 3: Melamine tautomery. Amine form *s*-triaminotriazine (I), and imine form *iso*-melamine (II) [U.S. Food & Drug Administration, 2015].

Melamine and cyanuric acid dissolve easily in hot water and the solubility to 20 °C water for each alone is in the range 2-3 g/l. Their complex, melamine cyanurate, figure 4 is yet much less soluble about 0.01 g/l [Tittlemier, 2010]. Similarly melamine can interact with the DNA T-base through hydrogen bonds and form a stable conjugate [Xing et al., 2013]. There are procedures to improve the solubility e.g. complex by adding acetonitrile as a co-solvent or breaking the complex by using dimethylamine in extraction. If this aspect is not taken care of the analytical results could have a negative bias.[European Food & Safety Authority, 2010] This would affect samples with very high levels of both melamine and cyanuric acid that was not the case for the Chinese milk incident. In the pet feed adulteration however the cyanuric acid content was higher [U.S. Food & Drug Administration, 2007].

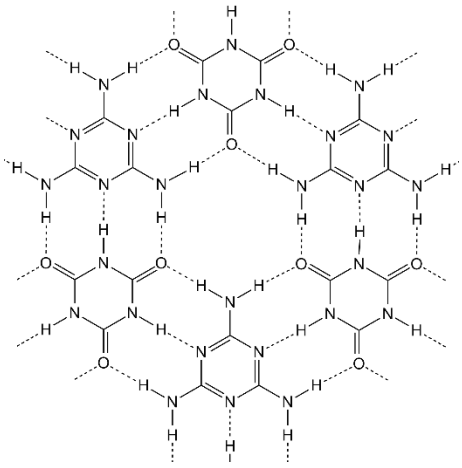
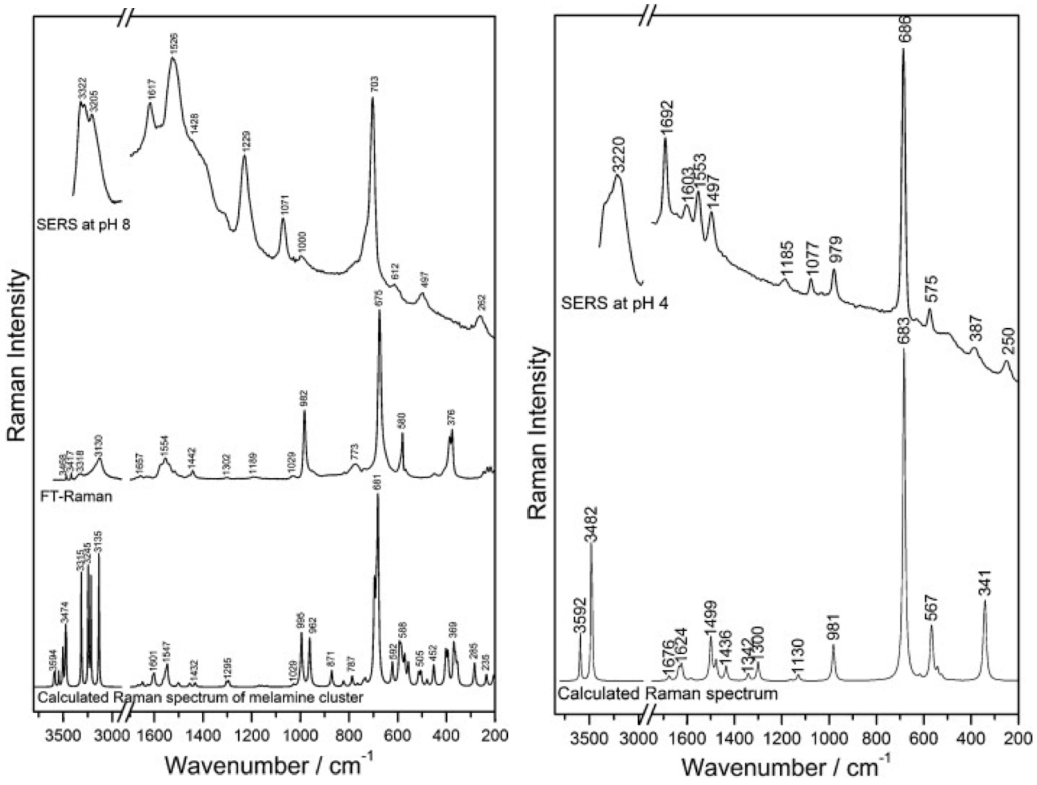


Figure 4: Melamine cyanurate complex.
[Texas A&M Unicersity, The department of Chemistry, Official Website, 2015]

2.4 Melamine SERS bands' chemistry

The SERS measurements for melamine can be done in colloidal suspension or dried in a solid state [Mircescu et al., 2012]. Since melamine is not water soluble enough, the achieved concentration in water was not high enough to record a good conventional FT-Raman spectrum of melamine in aqueous solution. The FT-Raman spectrum differs from SERS and therefore can not be used directly as a reference. Depending on experimental set up also the SERS spectra have differences that ought to be recognized.

In the FT-Raman spectrum of melamine powder in Figure 5a the most intense bands are found are assigned to the following vibrations of molecular bonds: 376 cm^{-1} CN bending, 550 cm^{-1} NCN bending and twisting of NH_2, band at 675 cm^{-1} the ring breathing and 982 cm^{-1} to tiazine trigonal bending of bonds CNC and NCN.



(a) Neutral molecule, pH 8. (b) Protonated molecule, pH 4.

Figure 5: FT Raman and SERS spectra of neutral species and protonated melamine compared with calculated Raman spectra [Mircescu et al., 2012]

The recorded SERS spectrum at pH 8 in water solution differs slightly from the FT-Raman. The characteristic bands shift as a result of interactions with silver colloids. The triazine ring breathing vibration is shifted higher from 675 cm^{-1} to 703 cm^{-1}. The trigonal bending of bonds CNC and NCN is seen higher at 1000 cm^{-1}. SERS enhances some bands that are not visible in FT-Raman. Vibrations from

ring stretching, ring in plane deformation and NH_2 groups' deformation are assigned to peaks at 1071 and 1229 cm^{-1}. The protonated melamine also has changes in the spectrum. The band at 686 cm^{-1} corresponding to the triazine ring breathing vibration is shifted higher respect to FT-Ramans' but not as much as with the normal molecule. The trigonal bending of bonds CNC and NCN are found lower, at 979 cm^{-1}. [Mircescu et al., 2012]

The frequency shift of the NH_2 wagging mode of aromatic amines adsorbed on a metal surface depends strongly on the hybridized property of the NH_2 group [Tao et al., 2013]. In the neutral melamine, the band was found at 1526 and in the protonated one at 1497 cm^{-1}. As the highest electron density in a neutral molecule is located on the ring nitrogens, thus the molecule is supposed to adsorb through these atoms leading to face-on adsorption.

Evenyn Kämmer *et al.* found that combining nanoparticles with a special activation agent is crucial for the observed SERS enhancement. Their study compares the change in peak height and area of two SERS bands. First of which is at 685 cm^{-1} corresponds to a ring breathing mode and an in-plane deformation of the triazine ring and the ring breathing 2 mode. The second, 990 cm^{-1}, is assigned to a ring breathing mode and an in-plane deformation of the triazine ring of melamine. The findings showed that enhancement of these bands was the best on spherical gold colloids of a diameter 120 nm when activated with KBr and UV excitation wavelength of 244 nm was used.

In Figure 6 the bulk melamine spectrum is being compared with SERS spectrum. The shift in the peak $\sim$ 700 cm^{-1} indicates to surface enhancement of the spectrum. The bulk spectrum was measured from crystalline powder and the SERS spectrum from a dried sample of 10 mg/l melamine in milk.

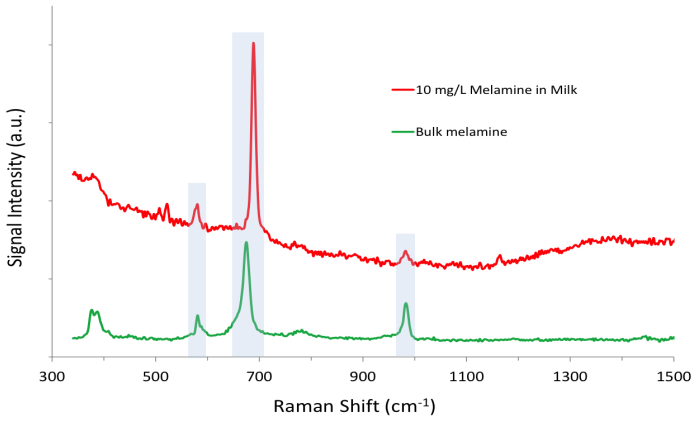


Figure 6: Melamine bulk Raman spectrum measured from dry powder and a SER spectrum in milk

2.5 Milk protein content measurements

The two most wildly used procedures to monitor milk protein content are the Kjeldahl method and the Dumas method. Kjeldahl is laborious but does not need high end instrumentation. Its other disadvantage is that all organic nitrogenous compounds in solution are detected. The competitive method for Kjeldahl is Dumas method that quantitatively measures crude protein content and is easily automated but has a high investment cost. Evaluation the bias between these methods, suggests to systematic error from methodology rather than measurement precision. [Wiles et al., 1997]

In short, Kjeldahl method determines the nitrogen content of organic and inorganic substances in solution by the titration of ammonia. The process has in principle three steps: Digestion, distillation and titration. First, nitrogen in organic samples is decomposed by boiling the sample in concentrated sulphuric acid solution resulting in an ammonium sulfate solution. Next step is the addition of a base allowing $NH_4{}^+$ to be distilled out as NH_3. Finally, the recovered distillate is titrated to quantify the amount of ammonia.

The weakness of the Kjeldahl method not being specific to nitrogen in proteins has for long provided a way to cover the watering down milk with various compounds for instance urea and melamine [Kumar et al., 2000].

2.6 Competitive methods for detecting melamine in milk

For quick qualitative detection several applications have been proposed such as Infrared reflectance [Liu et al., 2011] [Jawaid et al., 2013], Colorimetric methods that use nanoparticles [Kumar et al., 2014], competitive ELISA [Garber, 2008] and SERS. Most of these techniques are qualitative or quantitative and require the preparation of a calibration curve. Manufacturers still advise analyzing positive test results further with HPLC or LC-MS/MS.

Commercial ELISA-kits' are available by at least Abraxis and Biosense Laboratories. One known assay for melamine detection with Enzyme-Linked Immunnosorbent Assay is horseradish peroxidase (HRP) conjugated melamine. The labelled melamine competes for the same binding site of an epitope of a primary antibody in the solution. The signal of the HRP-label is eventually measured with microplate photometer using 450 nm wavelength. As the competitive binding is concentration dependent the signal in inversely correlated with the amount of melamine in the sample. The result of the signal intensity is referred to a standard curve to find the concentration in the sample. For strongly positive samples, several two-fold dilutions were used to construct the standard curve. Positive results are guided to be analysis further with LC/MS. The method is relatively fast but a calibration is required and the measurement procedure itself is not entirely trouble free. The cost of AgraQuantő Melamine Sensitive from ROMER Labs Diagnostic GmbH is 500 EUR + shipping. Abraxis Melamine ELISA Kit from Biosence Laboratories AS costs 488 EUR per kit + VAT and shipping.

Colorimetric detection has no commercial application yet however the preliminary findings of Kumar *et al.* 2014 seem promising. The detection they proposed relies on the color change of solution when nano particles cluster due to binding with melamine. The change in absorbance measured with (UV/Vis) spectrometer correlates with the concentration. The method requires filtration as a pretreatment and the binding process at low concentrations, 1 mg/l, slows down but results were obtained within 15 min. [Kumar et al., 2014]

Competition within SERS technologies exists too. Melamine detection has been attempted with various silicon substrates with promising results still without a commercial breakthrough. Substrates as P-SERS paper strips by Diagnostic anSERS are already available but to my knowledge not wildly used.

3 SERS Substrate

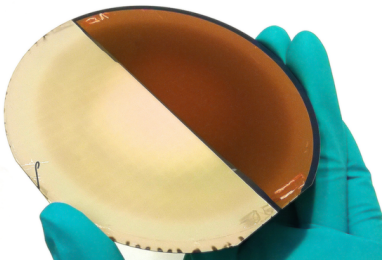


Figure 7: Silicon NP wafer 50:50 Au/Ag.

The SERS-active substrates used in this work were silicone and fused silica nanopillars. 200 nm coating of both gold and silver were deposited on the same wafer, as the figure 7 illustrates, to minimize error caused by wafer to wafer differences in nanostructures. Originally, the substrates of silicon and fused silica nanopillars were to be compared and the better performing type to be used in further experiments. However, the fabrication of the fused silica wafers was delayed and reliable comparison between the two could not be completed. Hence the majority of results presented are on gold coated silicon substrate.

3.1 Silicon substrate fabrication

The nanopillar fabrication illustrated schematically in Figure 8 is in fact a four-step process. Firstly, a maskless reactive ion etch, RIE, where no lithographic step is needed. After etching the pillars are cleaned with oxygen plasma treatment and then annealed at high temperature. Lastly the metal coating is deposited with an e-beam evaporation. Fabricating these substrates is possible in almost any silicon processing facility with a relatively low cost [Schmidt et al., 2012]. In practice obtaining the same structures requires careful control on all processing steps. The Si5003 was fabricated by Michael Stenbæk Schmidt and had a slightly thicker gold coating, 225 nm. Fabrication of batch SiLT2 was performed following given process flow as part of the thesis work. Silicon wafers used in this project were 4" p-type (boron), single side polished (100) wafers.

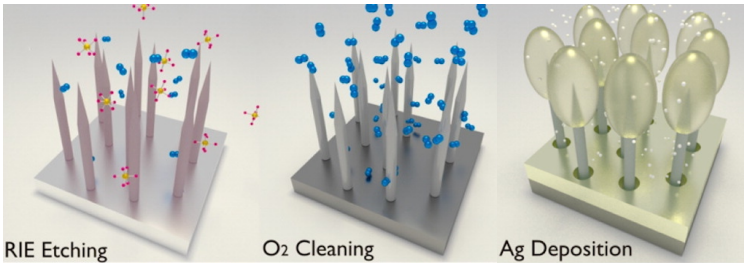


Figure 8: Schematic fabrication process of the silicon nanopillars: Silicon reactive etching, O_2 cleaning and e-beam metal deposition. [Wu et al., 2015]

The maskless reactive ion etch (ICP) RIE process utilizes ion filled plasma. The ions etch the surface when accelerated towards it by a magnetic field. Plasma etching is a chaotic combination of chemical and physical processes in which cations are produced from reactive gases, accelerated to the silicon surface where they chemically react with it. Crudely, physical etching is a sputtering-like event that utilizes ions accelerated to the surface. Chemical etching results in reaction of silicone and radical gas. [Franssila, 2010]

$$e^- + SF_{6(g)} \longrightarrow e^- + SF_5^{\bullet} + F^{\bullet}_{(g)}$$
$$4\,F^{\bullet}_{(g)} + Si_{(s)} \longrightarrow SiF_{4(g)}$$
$$O^{\bullet}_{(g)} + Si_{(s)} + F^{\bullet}_{(g)} \longrightarrow Si_xF_yO_{z(s)}$$

Etching was conducted in an Advanced Silicon Etcher (ASE, Surface Technology Systems MESC Multiplex ICP) Process gases used were SF_6 and O_2. The etching process was finalized with an oxygen plasma treatment with O_2. [lähde]. The etched wafers were annealed before e-beam metal deposition.

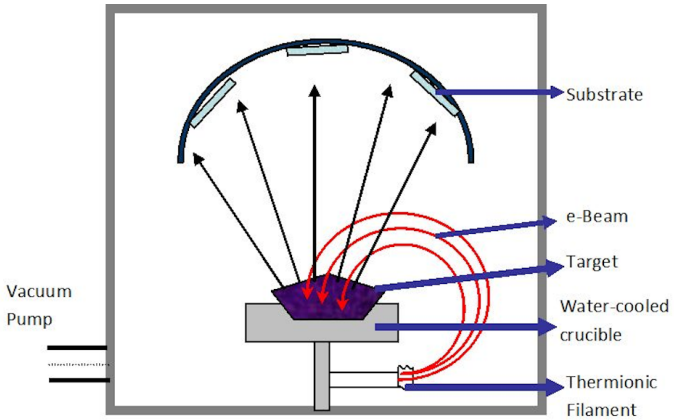


Figure 9: E-beam precess, schematically. [Gupta et al., 2013]

The first half of the wafer was coated in electron beam evaporation of silver using Alcatel SCM 600. The metal layer thickness may vary within $\pm$ 5 nm. The same thickness of gold was then deposited at equivalent rate and chamber pressure with Wordentec QCL 800. As seen, respectively, in the figure 8 the deposited metal sits on the tips of the pillars but in addition the that the ground around pillars' stalk gets metal coated.

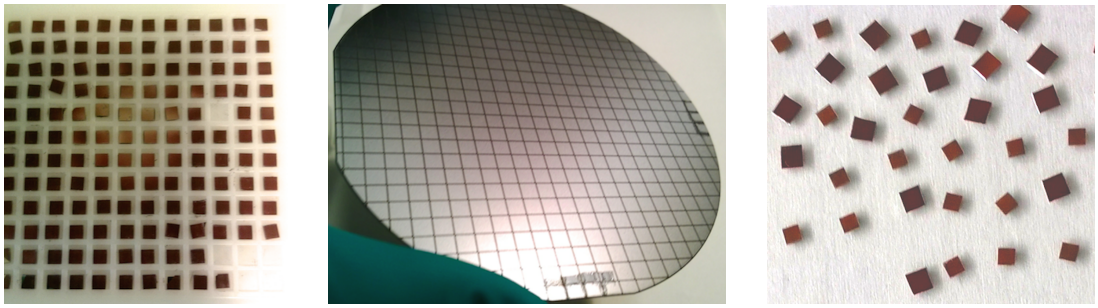
3.2 Fused silica substrate fabrication

The biggest difference in silicon and glass fabrication process is the gas selection. Glass is a very non-reactive and stable substance hence, a higher sulfur hexafluoride flow rate at higher temperature. Despite all the effort to fabricate fused silica wafers for this work only two glass wafers were used. The wafer, ID *BN9np* was fabricated by PhD candidate Anil Thilsted and *Strauss* by Julia Cathrine Dyrnum as part of her B.Sc thesis project. The fabrication process differs from silicon fabrication and is known to be prone to variation in nanosrtuctures between fabricated wafers. The variations may be explained by the operational instability of the instrument in use.

To ensure a firm enough clamping of the wafer a layer of aluminum was deposited on the top of the fused silica wafers. The reactive oxide etch was done with the Advanced Oxide Etcher, (AOE, STS MESC Multiplex ICP) and no annealing was needed before metal deposition. The e-beam process is carried on as with the silicon substrate described above.

3.3 Wafer dicing

The wafers were diced to 4x4 mm chips in order to gain similar SERS samples, while easing the wafer handling. Equipment used was Laser Micromachining Tool microSTRUCT vario, 3D-Micromac AG. A grid was machined on the backside of the wafer to prevent the heat destroying the nanostructures. The fused silica wafer *BN9np* needed to be diced with red, 1064 nm laser whereas silicon wafers were diced with the green 532 nm laser. Silicon substrates were laid on a rubber o-ring for the dicing and taped on a steel plate to prevent misalignment during dicing. The residues of tape were removed carefully after gently bowing away the dust created by machining on the backside of the wafer.



(a) *BN9* diced. (b) *SiLT2* machinined. (c) Si-chips 4x4 & 5x5 mm.

Figure 10: Laser dicing of chips.

Glass being amorphous the wafers needed to be diced almost through to break it. The glass wafer was held in place by a thin plastic film from the backside during the manufacturing. The laser naturally burned the plastic film and produced soot that was mostly removable by blowing compressed air gently from the back side. The larger amount of dust and soot created in the fused silica dicing may have decreased the performance of the substrate and are also a contamination risk when the chips are in contact with the sample solution by immersion. Before the SERS measurements the film residues were gently removed as the chips had completely dried the backside was therefore clean from sample solution that helped focusing with scratches on the top of the chip.

With a good alignment of dicing and the direction of the silicon lattice, the wafer could be broken along the dicing and less time was needed for machining than with the glass wafer which is an advantage as contamination can be avoided a little better. Additionally differences in dicing produces different edge in glass and in silicon chips. Silicone breaks in the direction of the lattice and the edges are sharp where as the fused silica edges are more often rougher. The glass wafer being also extremely fragile it could not be storage as whole and was thereby diced directly after machining and stored in a holder shown in the figure 10a *in vacuo* to prevent from exposing the substrate to air. The color difference between batches may arise from thicker gold layer or differences in nanograss. The completed silicon wafers were diced as required to prevent mechanical damage caused by the contact with the lid of the holder. Silver substrates were also protected from light by a wrapping with foil.

3.4 Substrate cleaning

The cleaning was done not only remove organic contaminants from the surface but also to make the substrate hydrophilic. Good spreading of water and was assumed to be beneficial for SERS detection. At the beginning of the study, the substrates indeed showed good spreading. However, in time the chips no longer possessed this ultra hydrophilic behaviour even after cleaning.

Substrate cleaning was first done by ozone plasma cleaning but due to high contamination risk at those lab premises the cleaning method was changed to heating the chips to 200 °C for 5 min on a hotplate after which they were let to cool down to 30 °C. The heating rate was 150 °C/min no active cooling was applied and cooling down took 19 to 12 minutes. The treatment changes the color of the chips to lighter russet. The heat profile of the cleaning is shown in Figure 11. The silver substrates were kept under nitrogen to prevent oxidation during heating. Gold does not form an oxide and therefore nitrogen atmosphere is not needed [White, 1964]. However the wetting of the surface was slightly better when chips were heated in air which indicates to reactions with silicon or some relaxation process in the coating that benefits the atmospheric conditions.

To minimize the contamination risk during the heating and cool down the hot plate was first wiper with deionized water and ethanol after which the system was heated to 300 °C for 5 min under N_2 flow that was turned on approximately one minute before the hot plate cleaning was started.

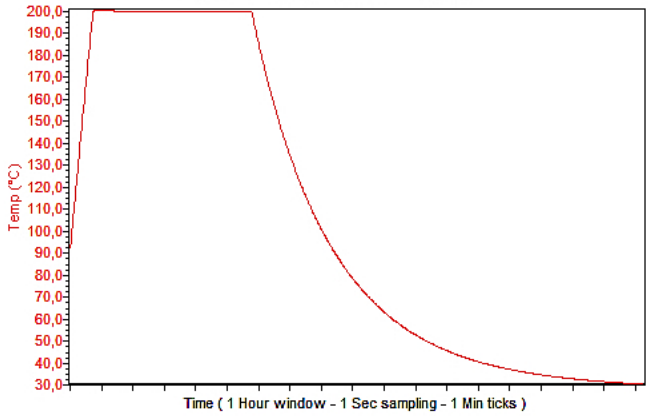


Figure 11: The heat profile of cleaning substrate chips for 5 min at 200 °C under N_2 atmorphere.

The choice of the cleaning temperature and time were based on experience. The effect of heat treatment at 300 °C and 200 °C was compared against performance

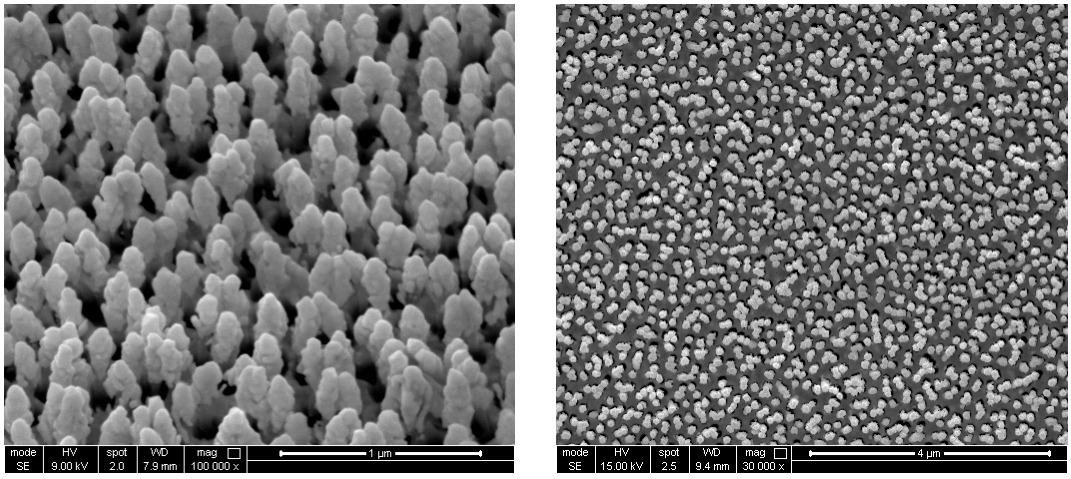
before the cleaning. The higher temperature treatment led to a hydrophilic surface but may have damaged some of the chips. That was seen as lower SERS performance when benchmarking the wafers 19. The effect of cleaning at high temperatures on SERS related properties such as surface roughness was not looked into. The wetting of the chips however is essential and the change in the spearing has direct impact on SERS measurements. The aging of the substrate was unexpected and at the time of the observations were made no time was left to examine it. For instance, the contact angle between water and nano-structured surface may be attributed to characteristics of the nano-structures. Both high surface roughness and surface porosity evoke ultra hydrophilic behavior [Dhumale et al., 2010]. With one month old gold substrates the droplets were pinning and did not roll off even when the substrate was tilted 90° and 180°. This change in wetting may have affected the results of this study and the aging of substrates requires further examination. The measurement of dynamic contact angle for instance would be easily arranged.

3.5 Quality control with SEM

SEM imaging was done at DTU CEN with Quanta FEG 200 ESEM and details of imaging can be found in appendix I.

3.5.1 Silicon nanopillars

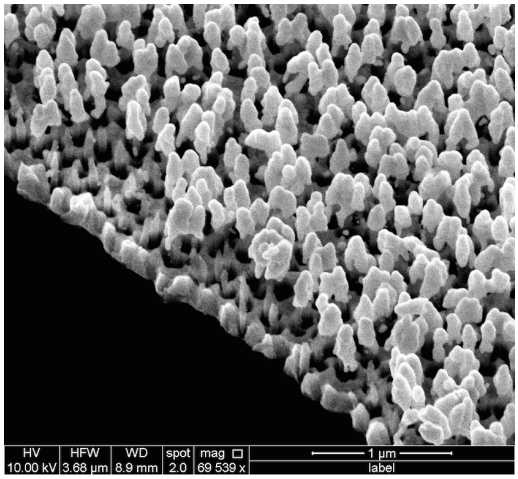
The images from each batch before heating as in figure 12 and when heated to 200 °C in the figure 13 show no signs of the wafer being damaged by cleaning. Also the surface seems rather clean and no significant deposition of dust was observed. The structures on the substrates chips were uniform through the entire chip area. An edge of a dried water droplet on *SiLT2 4* in Figure 14 shows how the pillars lean together forming hotspots.



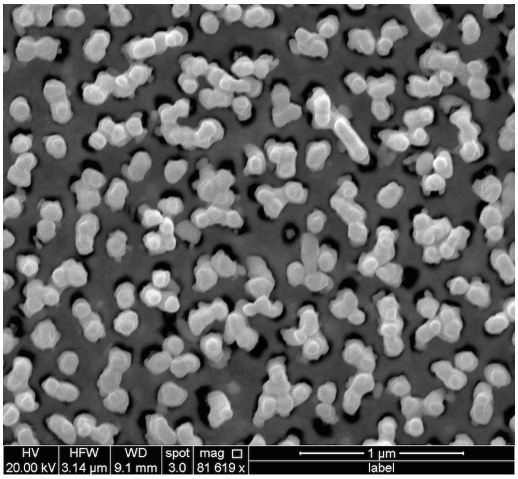
(a) Not heated 40° tilted.

(b) Not heated, top view.

Figure 12: *SiLT2 4* Au the substrate was stored *in vacuo*. No heat treatment to clean the substrate.



(a) 38° tilted.



(b) Top view.

Figure 13: *SiLT2 3* Au, 5 min at 200 °C under inert gas. The gold coating is seen as a lighter phase on the tips of the grass and also on the "ground" around the pillars.

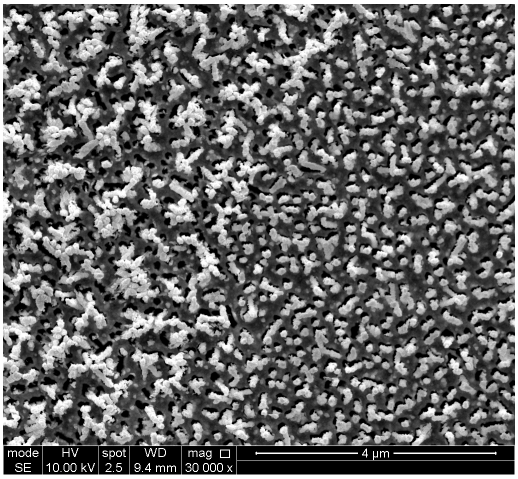
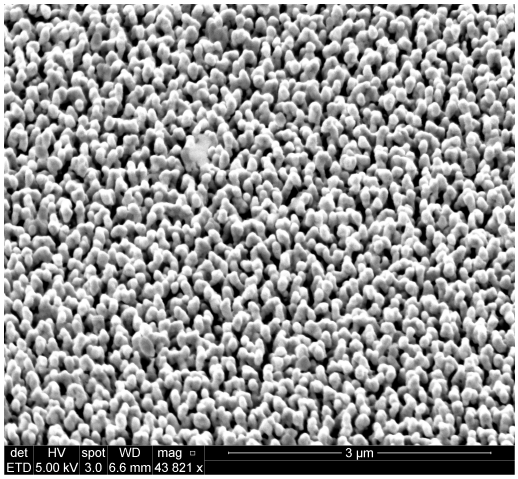


Figure 14: Hot spots where the nanopilllars lean. A dried water droplet edge on *SiLT2 4* Au substrate. Cleaned 5 min at 200 °C under inert gas.

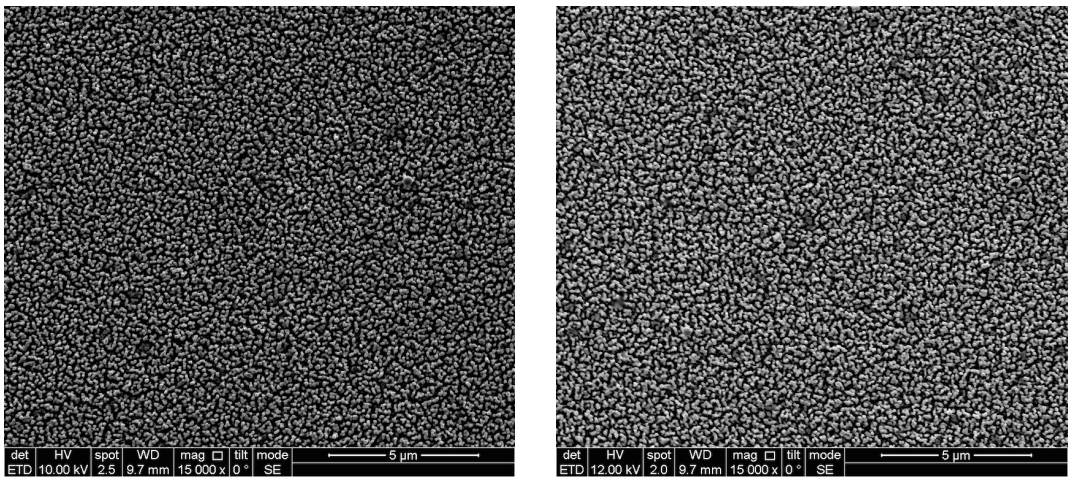
3.5.2 Fused silica nanopillars

The chips for SEM imaging were selected from the outer 10 mm zone of the wafer to compare the cleaning and leaning prior to the characterization of differences in structures variation within the wafer. The images gained show that the fused silica pillars fabricated do not lean like silicon nanopillars.



(a) Clean chip. (b) Water on the substrate.

Figure 15: *BN9np* cleaned by O_2 plasma cleaning 35 s



(a) No cleaning, the chip stored *in vacuo*. (b) 200 °C 5 min cleaning.

Figure 16: Water on *BN9np* substrate with no heat treatment (a), and when cleaned by heating to 200 °C for 5 min (b).

3.6 Wafer benchmarking

The SERS substrates were benchmarked with bio-molecular grade water and 100 µM BPE/EtOH solution. BPE molecular structure is illustrated in Figure 17. The 2 mM stock solution final volume was 45 ml so the weighing error was kept under 1 % as seen in the graph 18. BPE was measured directly in a 50 ml centrifugal tube where ethanol was added directly. The stock solution was prepared the same day and diluted after $\sim$ 5 min of stabilization. The evaporation rate was assumed negligible.

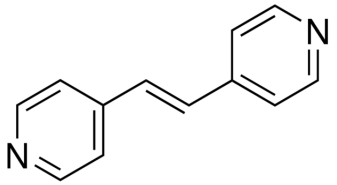


Figure 17: BPE molecular structure cas: 13362-78-2. [Sigma-Aldrich MSDS, 2015]

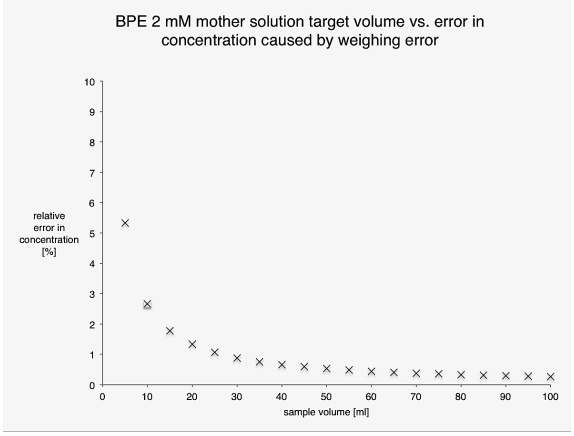
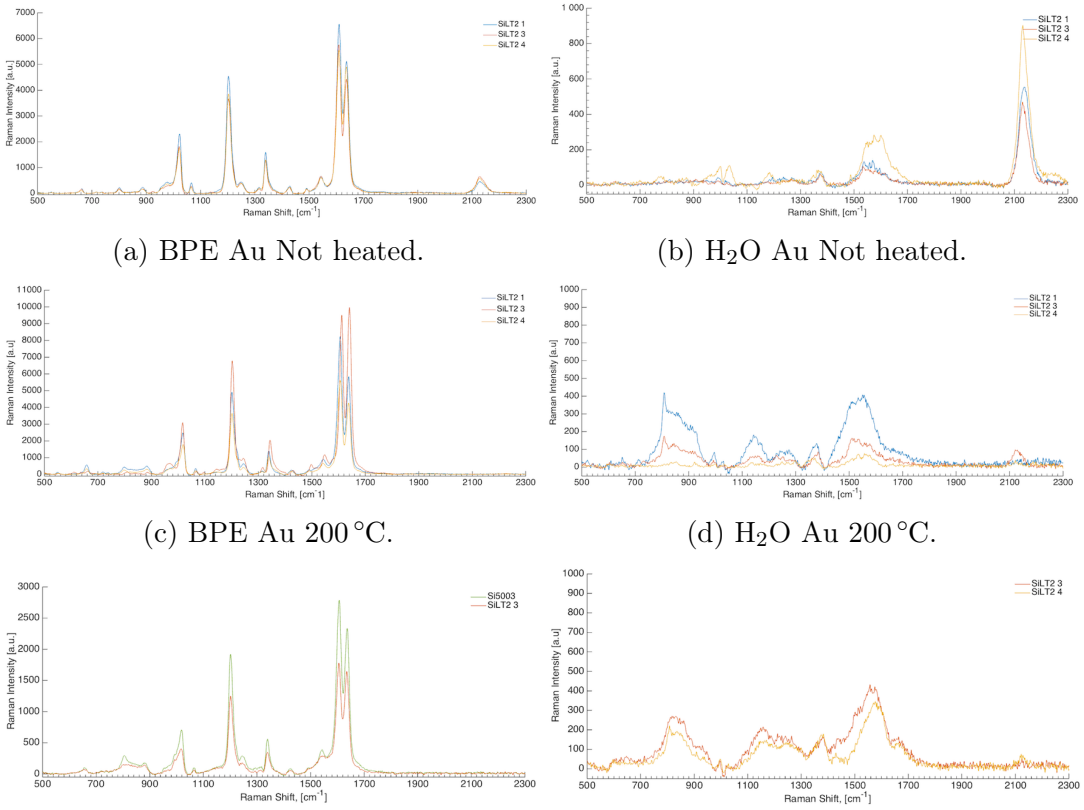


Figure 18: Relative error in 2 mM solution concentration arising from the weighing error. The balance accuracy was $\pm$0.1 mg.

The benchmarking does not follow the best possible practice and to obtain results that could be compared the benchmarking ought to be done routinely alongside the measurements. Moreover, the individual chips should be traceable to the location where they were cut from the wafer.

Two chips were chosen from each wafer to compare the performance of the metals and the wafers. By the time of bench marking the batch SiLT2 was already one month and Si5003 several months old but stored *in vacuo*. 5 µl droplet was pipetted on the chip and let to evaporate. The evaporation at room temperature took some seconds respectively. The SERS signal was collected from different points within the dried droplet. Difference in the signal from the drop edge or the centre was not looked into. One reason behind this decision was different solvent and that the ethanol wetted very large area, even the whole 4x4 chip. Systematic collection

respect to the direction of drying was difficult to arrange because ethanol leaves the substrate surface only slightly darker than dry areas and the centre might have been challenging to distinguish. The effect of cleaning at 200 °C and 300 °C was looked into by comparison against chips that were not cleaned.



(a) BPE Au Not heated.

(b) H_2O Au Not heated.

(c) BPE Au 200 °C.

(d) H_2O Au 200 °C.

(e) BPE Au 300 °C.

(f) H_2O Au 300 °C.

Figure 19: Silicon wafer benchmarking.

The least noisy background was obtained with chips that were not cleaned by heating. The background of the heated chips was very similar and but the higher temperature had a negative effect of BPE detection. The SEM imaging however did not reveal damaging of the substrates.

The SERS performance of the glass substrate was expected to vary greatly from the center of the wafer towards the edge of the metal ring. Therefore the benchmarking was performed by scratches aligned in straight line from the middle of the wafer to the metal ring 15, 20, 25 and 30 mm from the centre of the wafer. 5 µl droplet was pipetted by the scratches and an array of 6 x 6 measurement points, with 10 µm step was collected by each scratch in the wetted area. The results of remeasuring the benchmarking spots on the wafer *Strauss* are presented in Figure 20. The freshly benchmarked wafer showed that the intensity of the peak is highly

related to distance from the center of the wafer. As the wafer was fabricated and benchmarked already and stored in atmospheric conditions only the change in SERS performance could be due to various reasons. Hence only the outer 10 mm area was used to compare the effect of coverage on SERS performance. The trend in the benchmarking of the same substrate in Figure 20 after storage in atmospheric conditions and the benchmarking of the freshly fabricated wafer show a similar trend. However, the intensity measured from the old surface is surprisingly higher. Contamination was ruled out by taking a background of the are before introduction of BPE. It is clear that the substrate had been aging and the performance had changed.

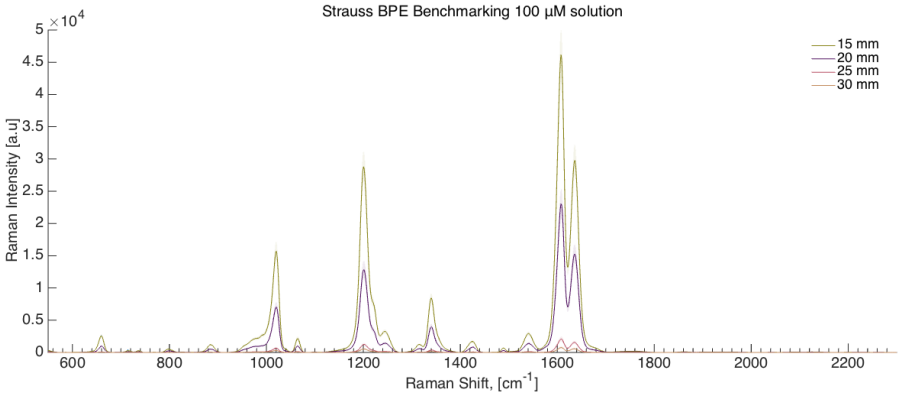


Figure 20: Benchmarking fused silica wafer *Strauss*, 100 µl BPE in EtOH. 15, 20, 25 and 30 mm from the center of the wafer.

4 Sample for SERS Detection of Melamine

Raw milk composition varies from farm to farm and through out milking season. "Raw milk offered for sale within and into the European Union has to be produced according to the requirements of Commission Directive 89/362/EEC and to meet quality standards described in Council Directive 92/46/EEC." [Hillerton and Berry, 2004]. These directives set microbiological limits to Salmonella spp, Listeria monocytogenes, Staphylococcus aureus, and E-Coli in milk. The demanded composition of milk varies between national quality classes which also determine the producer prices. The classes are set basically by microbiological limits, protein and fat content. Table 1 lays out an guide-lining composition of fresh milk. However, information on the lowest quality of milk bought by diaries was not easily available and is likely to vary greatly within the industry.

4.1 Milk - a complex colloidal solution

Milk is a complex chemical solution with maximum stability at 37 °C and pH of 6.5 to 6.7. Depending on the component of interest milk is an emulsion, a colloidal solution or a true solution. Milk fat globulins consist of milk fat lipids, fat soluble vitamins and form emulsion in water. Casein micelles are the size of colloids. Lactose and water soluble minerals and proteins dissolve forming a true solution.

Table 1: Respective composition of fresh milk sold to finish customers announced by EVIRA.

Component	**Comopsition % by weight.**
Water	86.7
Total solids	13
Fat	4.2
Proteins	3.5
Lactose	4.9
Minerals	0.7

Processing of milk aims at the production of different dairy products and expanding their lifetime. Figure 21 presents a simplified flowchart of dairy processing of fresh milk. It is evident that finding a single one sample preparation that would be applicable to all processing streams is highly unlikely. Moreover sampling milk of different quality classes may also require adjustments in sampling process and sample treatment. This work has been carried out using semi-skimmed ultra heat treated milk. An end product is likely less prone to variations from farm to farm but seasonal fluctuations can not be out ruled.

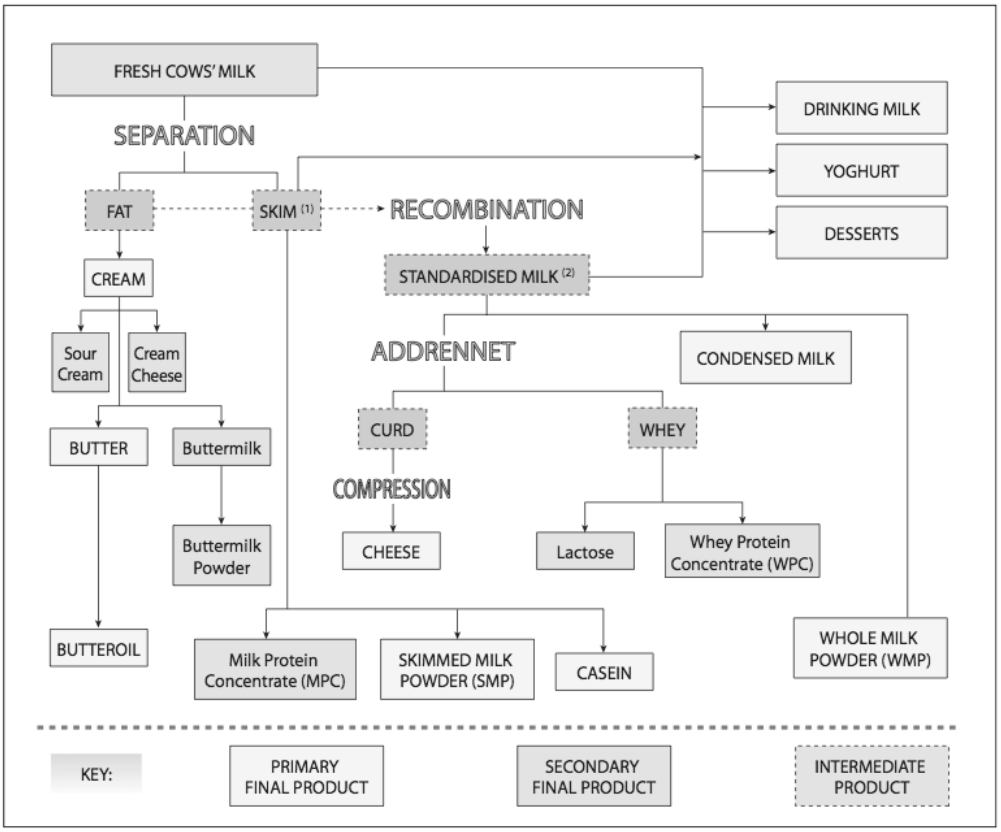


Figure 21: Raw milk is used in various dairy products and food ingredients that differ drastically in composition. [European Communities, 2006]

By skimming, thermal treatments and homogenizing the stability of milk is enhanced and bacteria killed. UHT milk, preserves long in room temperature maintaining better the colloidal structure than raw milk that creams and consolidates. This is because of the homogenisation that reduces the fat globulins size to roughly 1 µm. As a result the separation is hindered since the rising of fat globulins follow Stokes Law. UHT treatment is done to sterilize milk by heating to minimum 135 °C for 1 to 2 s. In the 1980s' UHT processing was found to cause the reactivation of phosphatase among some other enzymes in milk and that certain bacteria were resistant to heat [Harper, 1981]. The common practise in Finland is to use longer time, and temperature range from 140 to 150°C.

Casein micelles

Casein proteins can be divided into three subgroups, α, β and κ casein that differ from each other by a few amino acids. Casein molecules have hydrophilic and hydrophobic regions. Therefore it acts as surfactant and stabilizes colloids, foam, and the emulsion.

Together with some minerals and enzymes casein forms micelles which size ranges from 50 to 250nm in diameter. The micelles are reactive and dynamic components in milk. It has been suggested that a micelle comprised of aggregates of protein around calcium phosphate nanoclusters that form a structure sufficiently porous to hold a considerable amount of water not only on the surface but in the interior too Figure 22 presents a high-resolution field-emission scanning electron microscopy image of a casein micelle supporting the theory or aggregated structure around CaP nanoclusters. Figure 22 presents a high-resolution field-emission scanning electron microscopy image of a casein micelle supporting the theory or aggregated structure around CaP nanoclusters.

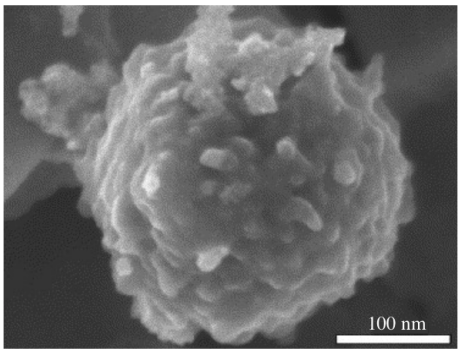
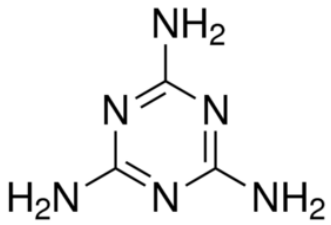


Figure 22: An electron micrograph of a casein micelle. [Dalgleish et al., 2004]

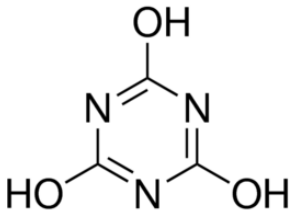
The more traditional model assumes small spherical casein sub-micelles of 10 to 15 nm by diameter to form large micelles of hundreds of sub-micelles.All models agree that the κ casein is mostly present as a stabilizing layer around the exterior of the micelle. It is know that the phosphoric acid in casein binds calcium and magnesium forming tertiary and quaternary structures. The calcium salts of α_s-casein and β-casein are poorly in water, while those of κ-casein are readily soluble driving κ-casein on the surfaces of the micelles [McSweeney and Fox, 2009].

4.2 Melamine in Milk

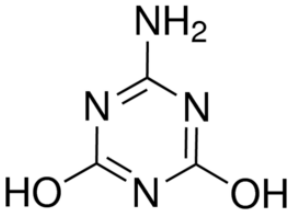
Melamine resin is wildly used in various plastics from laminates to kitchenware due to its capability for enhancing thermal durability and fire resistance. Melamine also gives the yellow colour to ink and melamine foam is common in insulating materials. Thereby it is produced as a high volume industrial chemical and depending on the purification process, the product may also have structurally related chemicals as cyanuric acid, ammelide and ammeline. Presence these substances in food can result from degradation from materials made of melamine-formaldehyde plastics. Also melamine has been found formed as a metabolite and degradation products of cyromazine, a plant protection product and a veterinary drug. However, the supreme cause for the presence of these compounds in milk and feed is adulteration as common protein tests can be falsified with an increase in nitrogen present in the sample.



(a) Melamine chemical structure, cas:108-78-1.



(b) Cyanuric acid chemical structure, cas: 108-80-5.



(c) Ammelide chemical structure, cas: 645-93-2.

Figure 23: Molecular structure of mealmine and its associates. [Sigma-Aldrich MSDS, 2015]

Structure of melamine is shown in Figure 23a. The molecule has 67 % by weight nitrogen, that has made it attractive substance to taint milk with. The addition of 1 g/l of melamine in milk increases the apparent protein content by 0.4 %. Melamine has higher solubility in warm water that allows has high concentration as 3.1 g/l of melamine in powdered milk products. Such addition may cause overestimation of the protein content in liquid milk by 30 % when using the Kjeldahl method. [Hau et al., 2009]

Melamine does not exhibit systemic toxicity, but is able to de complex with other substances such as endogenous uric acid or cyanuric acid to form crystals in the urine, which cause kidney damage. Studies on the renal toxicity of melamine with rodents, sheep and dogs, showed formation of urinary stones that may propagate to kidney failure. Thereby melamine Tolerable Daily Intake (TDI) is set to 0.2 mg/kg body weight [European Food & Safety Authority, 2010] and the Food and Drug Administration (FDA) have responded by stating that melamine levels at or below 1 ppm in infant formula do not raise public health concerns. [European Food & Safety Authority, 2010]

Melamine forms an insoluble salt with Cyanuric acid at acidic pH. Both have been found added in pet feed in the USA [European Food & Safety Authority, 2010] . Depending on the purification process of melamine Cyanuric acid and Ammelide are also present in adulterated milk. The sensitivity of the detection towards melamine or melamine-like compounds ought to be tested [Braekevelt, 2011]. The complex media and capability of binding with hydrogen bonding to some proteins make the detection of melamine at low concentration challenging.

5 Sample Preparation Schemes

The starting point of this work was to find a sample preparation method for field device SERS and such device may benefit from sample preparation optimized for different milk types. The challenges in measuring low concentrations in complex media as milk are firstly how to obtain distinguishable spectra, secondly the sampling statistics and thirdly the reproducibility of the sampling and detection.

In previous attempts to detect melamine in milk drops the characteristic peaks were found only in a very narrow area in the edge of a droplet of untreated milk injected on the substrate. In the sampling chain, the droplet itself causes a statistical problem which ravels around segregation due to the heterogeneity of milk. The drying process of the droplet produces a varying deposition rate of substances on the chip. As it is impossible to make identical droplets, it is nearly impossible to estimate the concentration gradient along the direction of drying in the droplet. In addition, measuring the melamine signal inside the droplet failed due to high noise and most probably decomposing material even with a low laser power.

Mineralized deposition and proteins are an issue in SERS measurements as the organic compounds may block the surface, give arise to noise and might also decompose during the measurement. Previously, melamine had been measured from milk droplets where the SERS signal was observable only at the edge of the droplet. The aim of sample preparation was of produce large areas where the melamine signal was visible and the background noise minimal.

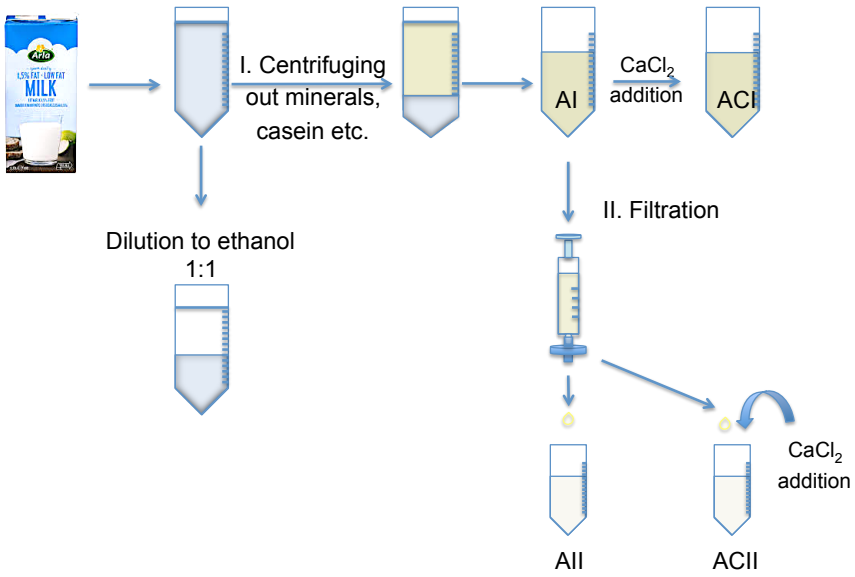


Figure 24: Sample preparation schemes.

Milk mother solutions were made from Arla 1.5 % UHT skimmed milk. These samples were stored maximum 4 days in fridge. In the 1980s' UHT processing was found to cause the reactivation of phosphatase among some other enzymes in milk and that certain bacteria were resistant to heat [Harper, 1981]. The changes in milk during the maximum 4 days storage was monitored only by blank reference samples. These control samples taken revealed no significant aging of the solution however it may have significance in interactions between proteins and melamine.

Table 2: Table 1 Samples

Sample ID	**Description**
H2O	Water at neutral pH
H2O A	Water at pH 4.5
Milk	Arla UHT Milk
AI	Centrifuged milk at pH 4.5
ACI	Centrifuged milk at pH 4.5. $CaCl_2$ added
AII	Centrifuged milk at pH 4.5. Filtered.
ACII	Centrifuged milk at pH 4.5. Filtered. $CaCl_2$ added.
1:1 EtOH/H2O	Dilution of melamine spiked H_2O to ethanol
1:1 EtOH/Milk	Dilution of melamine spiked milk to ethanol

To overcome the segregation issues sample preparation was introduced. The chosen pretreatments of milk samples followed five preparation procedures, presented in Table 2. Melamine concentrations of 1 mg/l 100, 50, 10 and 1 mg/l were diluted from 1 mg/l and 50 mg/l stock solutions. Blank milk samples were assumed not to have melamine in them as melamine in Danish milk have not been reported. Reference measurements were taken with equivalent melamine concentrations in ultra pure water, MilliQ 18.2 MΩ at neutral, acidic pH and 1:1 EtOH/H_2O solution. 1 M hydrochloric acid was used to adjust the pH.

Melamine stock solutions were made to high enough volume to suppress change in concentration below 1%. Sources of errors in stock solution preparation are measuring errors from weighing and pipetting or syringe glass. The latter two were evaluated insignificant compared with the weighing error. The change in solution concentration caused by weighing error alone is shown in appendix I. Also melamine powder mass change due to humidity in the air is probable even thought melamine is not strongly hydroscopic. Thereby no drying was done prior the weighing. The solutions were all made by the author and the operator fingerprint can be expected to be the same in all samples.

The control samples with known end concentration were made by spiking 10 ml of the pretreated sample with melamine to 1 g/l concentration. The melamine baring stock solutions of each sample preparation scheme and their blank solution for diluting were let of stabilize over night in a fridge. The following morning dilutions of 1, 10 and 50 mg/l were made to final volumes of 10 ml. This aging of the once warmed up but acidic solution may have been exaggerated and can even bias the detection. The dissolution into acidic samples was rather quick and easy to see from the clear solutions. Yet the same waiting time has been used as with milk samples where the dissolution could not be estimated as easily.

5.1 Acidic sedimentation

The first sample preparation scheme was to sediment out minerals, proteins and fats to an extend that minimal amount of SERS substrate surface would block. Following that spectra could be gathered from randomly chosen parts of the chip and more homogeneous spreading of the sample would allow larger mapping area.

In acidic pH the casein micelles become less stable and eventually at the isoelectric point these proteins aggregate forming coagulum. The impact of pH on micellar structure in illustrated in Figure 25. At pH $\sim$ 4.6 casein will gradually precipitate but relatively little of other milk proteins will flocculate [Anjana et al., 2010]. Depending on the final pH and other components in the solution, the aggregates compose of casein as its salt form, isoelectric state, or both. By centrifugation, a precipitate of 20 % by vol. was taken off from the sample. This fraction was not analysed further. The liquid fraction recovered was slightly turbid yellow with protein like insoluble residues.

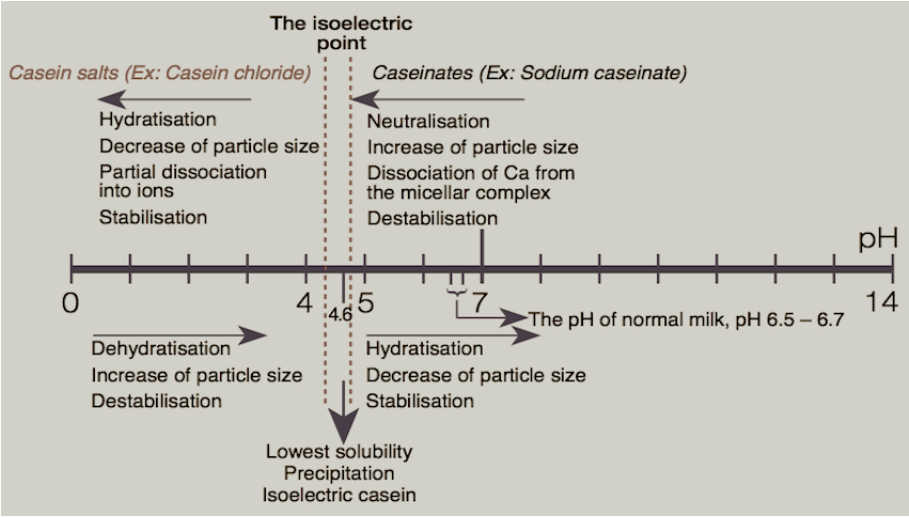


Figure 25: Casein micelles stability dependence on pH and $Ca_2{}^+$ ions concentration. [TetraPak, 2003] Chapter 2 p.13

Adjustment of pH below the isoelectric point of casein and centrifuging the precipitates bared a possibility of losing melamine with the discharge. Moreover, as melamine is often added with cyanuric acid there is a chance of obtaining insoluble salt at acidic pH too. Formation of melamine cyanurate is known to take place at pH 5 and increasing pH to 6 dissolves it [Grases et al., 2009]. Risk of significant losses of melamine when it is associated with cyanuric acid should be evaluated though the concentration of cyanuric acid in adulterated milk. For a rough estimate of melamine losses in centrifuging and filtration, the pretreated samples SER spectra were collected having added melamine in milk before and after the treatment aging processes. However lacking knowledge in analytical error these results have very little value. A mass balance on sampling was to be conducted by running tandem mass spectrometry, however this was ruled out for lack of time. Furthermore, the

evaluation of separated fractions might enlighten the desirable solution content for SERS measurement.

AI and ACI

UHT milk pH was set carefully to 4.5 with 1 M HCl at room temperature. The sample was divided into 45 ml sub-samples and centrifuged 15 min with 7500 rpm separating out a sold fraction of approximately 20 % by vol. The aqueous fractions were combined and the pH was rechecked after which the sample was split in fractions A and AC. The fraction AC had $CaCl_2$ added to 20 mM concentration. As there is a strong dependence between casein micelle size and Ca^+ ion content the addition was expected to increase the micelles size and to deactivate casein.

AII and ACII

Centrifuging the samples in 3 cm wide tubes also separated a top fraction of proteins and fat. The collected fraction was turbid light yellow liquid with two phases. Still the proteins were not well enough separated to be collected. Therefore a two-step filtration was conducted to to evaluate the possible unfavorable effect of these proteins and residual solids. Samples II were filtered with 0.45 µm nylon and 0.20 µm cellulose acetate 25 mm disk filters. Better filtration could be achieved by changing the nylon filter as it clogged easily. Also the filtration temperature could be adjusted. The changes in solution temperatures were relatively small and due to the long centrifuging time. The temperature of UHT milk during the $\sim$ 1.5 h treatment stayed between 19 and 24 °C. The heating of the milk could possibly be beneficial as an increase in temperature quickens the sedimentation and filtration.

5.2 1:1 EtOH dilution

The melamine bands observed in water solutions are rather weak and the substrates were known to give out stronger enhancement with other solvents. The improved performance may arise from spreading and drying of the sample. Thereby dilution of melamine spiked milk to ethanol, acetone and iso-propanol was tested at relatively low concentration. Based on preliminary experiment on silicon and fused silica substrates, further study with EtOH were taken. Both acetone and iso-propanol led to similar sedimentation in milk and the difference between these three was not drastic enough to out rule any in possible experiments in the future. The change of solvent also gave an option to avoid working in pH range where melamine cyanurate is stable. Simultaneously, the dilution created a demand for half lower detection limit on the substrate.

The dilution series were always made before the addition of ethanol. The aggregation could have biased pipetting due to strong segregation and settling of a solid phase. Right before introduction to the chips the mixture was shaken. Obtaining similar deposition was extremely difficult and the aggregates break when stirred, shaken or mixed in a vortex finder. The particle size created surely affects the segregation and detection. Another attempt to enhance the signal would be an ethanol addition on the chips after the milk sample deposition.

6 Results

6.1 SERS instrumentation

The SERS measurements were performed at DTU Nanotech with Thermo Scientific DXR^{TM} Raman Microscope, relevant operation parameters are listed in Appendix II. The instrument consists of a 780 nm wavelength (high brightness, NIR) frequency-stabilized single mode diode laser with a 50 cm^{-1} Rayleigh rejection filter, an aperture slit size of 25 or 50 µm and standard grating providing 50-3300 cm^{-1} spectral range with 5 cm^{-1} nominal resolution, (FWHM). Alignment and calibration were performed according to manufacturers' instructions before taking measurements. The setup also provides a bright-field illuminator with 10X and 50X microscopy objectives. All samples were inspected and sampling spot chosen either semi-randomly still avoiding spots where the chip was held by tweezers and right at the edges or selectively on features of interest.

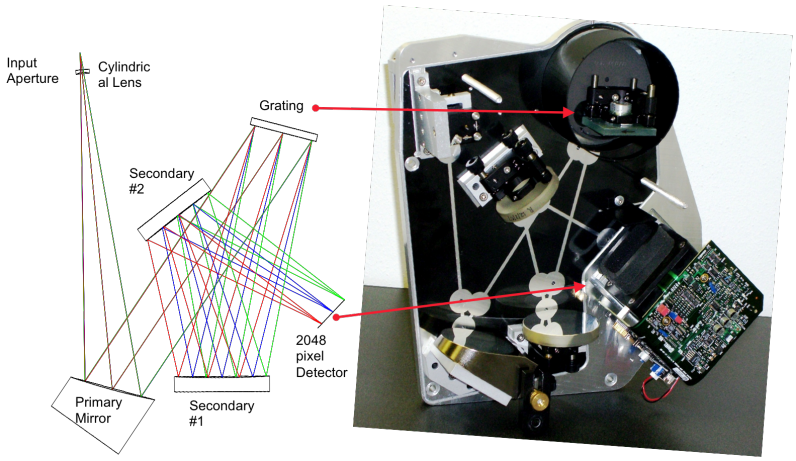


Figure 26: DXR spectrograph.

$$Resolution \propto \frac{w}{n\lambda} \tag{1}$$

$$n = \text{grating pitch, 400 (lines/mm)}$$

$$w = \text{CCD pixel size, 25 µm}$$

Sample exposure time and number of exposures both reduce noise. The longer exposure time allows the signal level to stay constant whereas numerous exposures reduce random noise by averaging the signal. Minimum amount of three exposures was used that also rejects cosmic rays. The exposure time was optimized together with the laser power due to fluorescence and photo-leaching of the sample. The maximum exposure time was found for the used laser power by increasing exposure until CCD overflow occurred. As the scattering intensity is proportional to $1/\lambda^4$

and fluorescence to one over the excitation wavelength, which indicates that shorter excitation wavelength is beneficial. Resolution, *eq.* 6.1, being proportional to the wavelength and inversely proportional to grating the pitch. The excitation wavelength selection is always a case sensitive and depends on the SERS substrate and the analyte. 633 nm laser might provide improved performance at the wavelengths of interest.

Spectra were collected with line scans over approximately 500 µm area. Larger mapping area were measured on 1 mg/l samples. The data analysis is further discussed in section Analytical methods. The data presented here is raw data as colormaps of single measurements. Spectra that bared no information, because of for instance fluorescence, were removed from the data. Average spectra were calculated only when concentrations were high enough to obtain the characteristic band at $\sim$700 cm^{-1} in nearly every sampling spot.

6.2 Melamine SERS measurements

At high concentrations, melamine forms large crystals from the edges towards the center of a drying droplet as seen in Figure 27. Around the dried droplet, a narrow slightly darker ring can be seen. The area is wetted but provided no or very weak melamine detection. When the chips were hydrophilic and dipped into the solution of melamine in equivalent concentration, crystals growth was not seen. Either is the crystallization reduced due to differences in drying or dipping reduced local up-concentration when the liquid film was thin. When the contact angle is high, the forming melamine crystals are allowed to float in the solution. This provides favourable conditions for crystal growth.

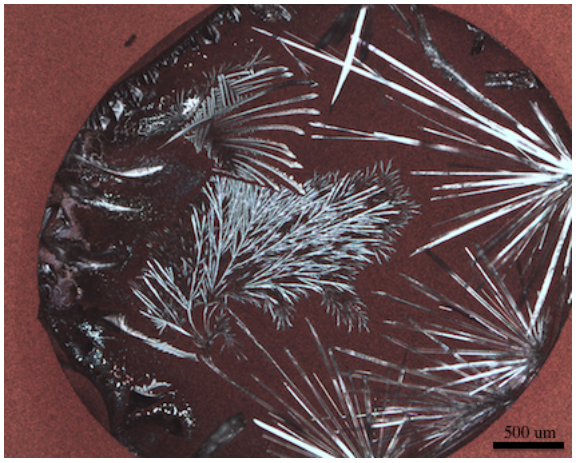
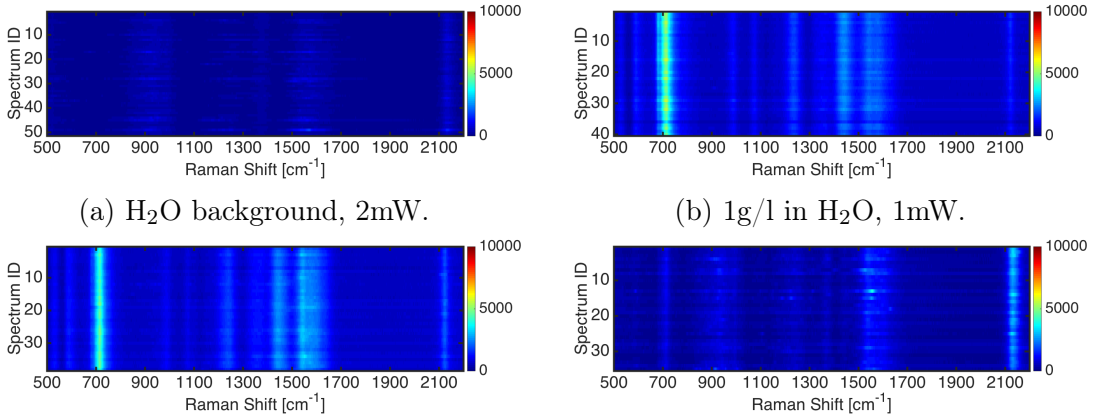


Figure 27: 1 µl drop of 1 g/l melamine in water dried on a SERS substrate, 10X magnification.

Reference measurements presented in Figure 28 were taken at three concentration, 1 g/l, 50 mg/l and 1 mg/l of melamine in water on Si-Au substrate.The colormaps present the collected SER spectrum ranked by the ID that are the ordinal number of measurement points.The data is baseline corrected and the color scale corresponds to the peak intensity. The background in water was relatively uniform and bands characteristic to melamine-like compounds were clearly visible in the colormaps, also at the set detection target of 1 mg/l. At high concentration 1 g/l the bands were found at $\sim$ 685 and 715 cm^{-1} which originate from the ring breathing 2 mode an in-plane deformation vibration of the triazine ring. The band at 685 is found in the bulk spectrum of melamine discussed in Section 2.4. It is assumed to originate from molecules that are not SER enhanced .

The band at 990 cm^{-1} corresponds to a ring breathing mode and an in-plane bending of bonds CNC and NCN deforming the triazine ring. It was only barely visible at 1 mg/l melamine in water. Raman intensity of the only band useful for characterisation, $\sim$ 685 and/or 715 cm^{-1}, shows to be also concentration dependent,

29. Still the observed band for 1 mg/l has over double the peak height of a measurement with ten times higher concentration. The difference might be explained by a combination of difference in the laser power and performance of the wafers.



(a) H_2O background, 2mW.

(b) 1g/l in H_2O, 1mW.

(c) 50 mg/l in H_2O, 1mW.

(d) 1 mg/l in H_2O, 2mW.

Figure 28: Melamine in water on Si-Au substrate.

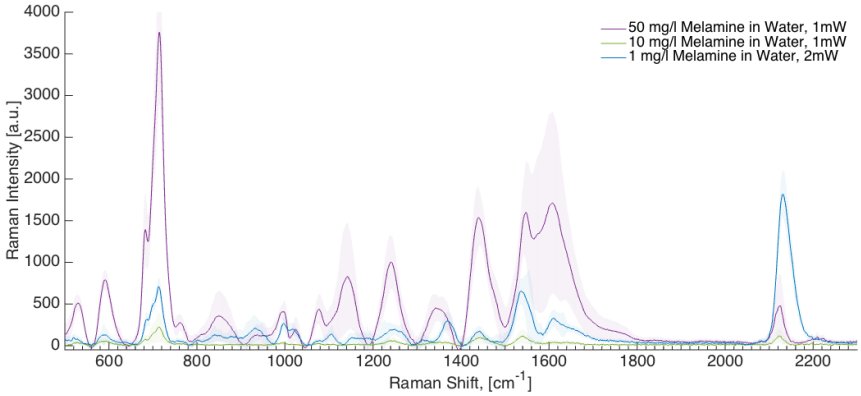


Figure 29: Concentrations of 1,10 and 50 mg/l melamine in water, baseline corrected average spectra of 45 measurement points.

The results from benchmarking already revealed quite drastic, even 50%, differences in peak intensity between wafer batches. The wafer to wafer difference in the peak intensity with gold coated silicon substrates also show clearly in the spectra in Figure 30. The findings are in agreement and 715 cm^{-1} band measured on Si5003 is approximately 60% of the one from SiLT2 1. Nevertheless, there is still dependence between the concentration and peak height.

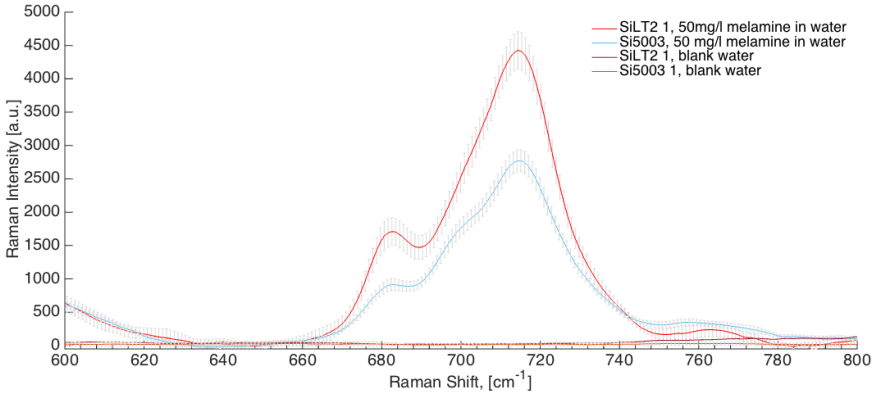


Figure 30: The change in the band $\sim 700\,\mathrm{cm}^{-1}$. Blank and 50 mg/l concentration with wafers from the batches *SiLT2* and *Si5003*. Baseline corrected average spectra of 45 measurement points.

The narrower slit widths, 25 µm, yields to greater spectral resolution but more signal is lost. To increase the amount of light that is being transmitted also wider slit size 50 µm was tested. The results were assumed to benefit detection in milk, where height resolution of the bands was not considered as crucial. The obtained spectra in Figure 46 shows how the slit width together with the laser power affects the band around $700\,\mathrm{cm}^{-1}$. An increase in the split width changes the ratio between the peaks at positions and unlike in previous measurements the peak height of $685\,\mathrm{cm}^{-1}$ peak is higher. Simultaneously, the standard derivation of the average spectrum increases drastically as expected.

6.2.1 Sensitivity towardsmelamine

Evaluation of sensitivity towards Melamine rises from two challenges. Firstly, if the peak observed originating form melamine or melamine like compounds and how similar ring structure can be mistaken as melamine. So, there is a risk of false positive if other compounds give rise to a peak at the same position. Secondly, this false negative result is possible if the solid crystals of melaminecyanurate are not visible in SERS or lost in sample pretreatment.

Ammelide and Cyanuric acid are often associated with melamine in milk and have very similar structure. Therefore parallel measurements were taken of these two and melamine in water and in milk at concentration on 50 mg/l. In practise the detection of melamine relies on the visibility of a peak $\sim 685\,\mathrm{cm}^{-1}$ and $715\,\mathrm{cm}^{-1}$ as discussed already in section 6.2.

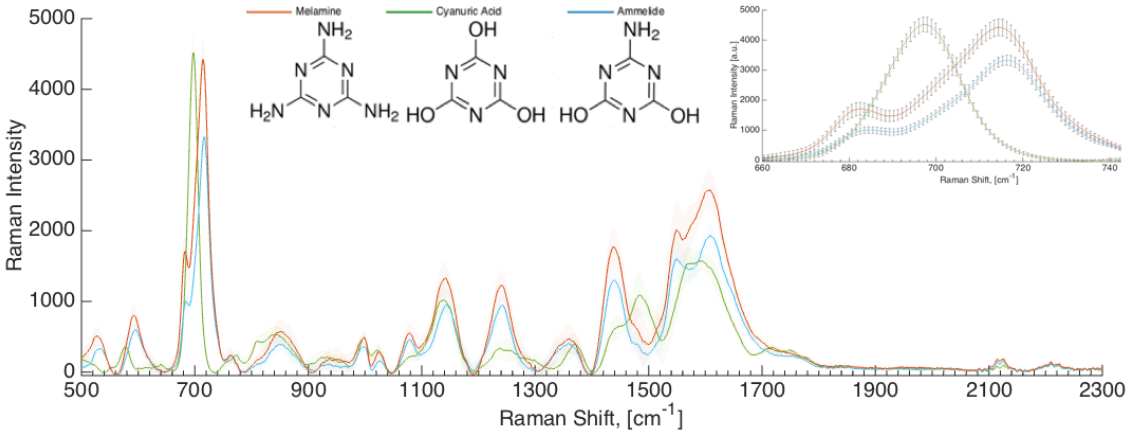


Figure 31: Melamine, Cyanuric acid and Ammelide in water. Collected on *SiLT2 1* substrate, 1 mW. Baseline corrected average spectra of over 50 measurement points.

6.3 Sample deposition and surface coverage

The microscope and SEM images of the samples were taken for the visual evaluation of deposited films. The information gained with optical microscopy, was used to compare pretreatment procedures but no further analysis on coverage was done. The few SEM images enlighten the sample spreading on the substrate. The micrograph are not directly linked to SERS results as the linking of the SEM and SERS measurement spot is not exactly simple.

The analyte was either pipetted directly on a cleaned substrate or the chip was dipped into 1 ml of sample solution for $\sim$ 3 to 5s. Excess material was let to flow off the chip by holding one edge against a clean wipe. The deposition of sample solution took place within 1 h from the start of the cleaning and the chips were kept under nitrogen. Measurements were taken on dried substrates within the same day and the chips were then stored in a fridge.

SER measurements on a milk droplet

The surface enhancement takes place at the hotspots and roughened metal surface. The sample however occupies a lot more area than the hotspots. Depending on the laser spot the gathered information originates roughly from bulky coagula, wetted areas with hotspots or non-deposited areas which are presented schematically in Figure 32.

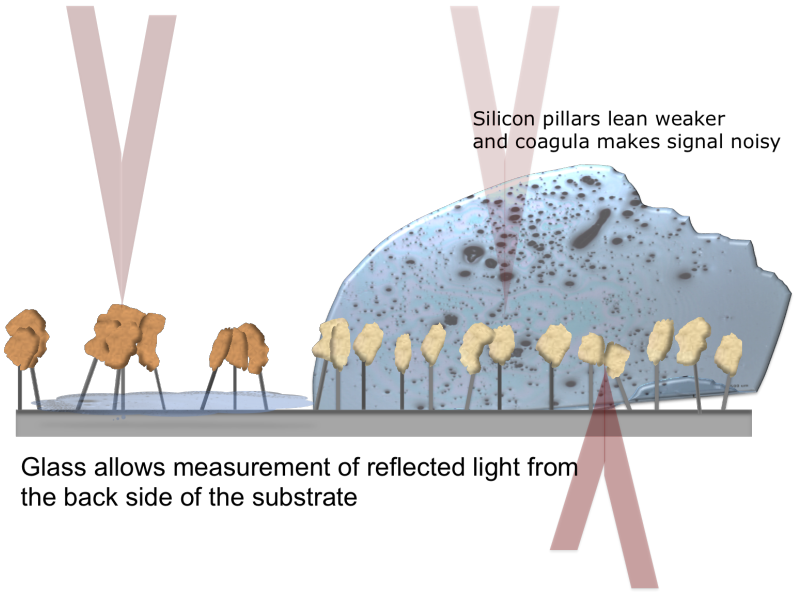


Figure 32: Milk droplet on substrate respectively.

Melamine has previously been measured from an edge of a milk drop in a very narrow area that may be found at the darkish boundary layer between milk clean substrate presented in Figure 33. Finding this area failed in a test where milk that was spiked with a high concentration, 1 g/l of melamine, was pipetted on clean substrate and let to dry on a hot plate at 30 °C. Line scans were run across randomly chosen spots at the edge with 10x magnification. The results of three scans were identical and were likely due to the spreading of milk on the substrate and the drying conditions. The collected spectra and the sampling spot are presented in colormap 34. The procedure was repeated with two more droplets without success.

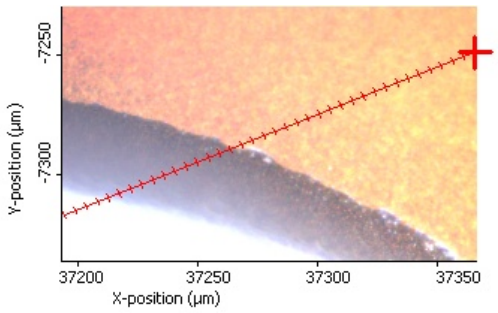


Figure 33: Milk droplet pipetted on substrate. Line scan from inside droplet (white) to wetted, but clean looking substrate across the edge. Marker corresponding the first sampling point, spectrum ID 38. Sampling interval 5 µm. 10X magnification.

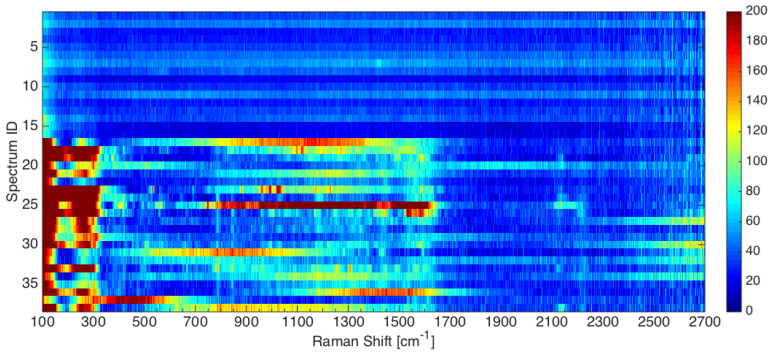


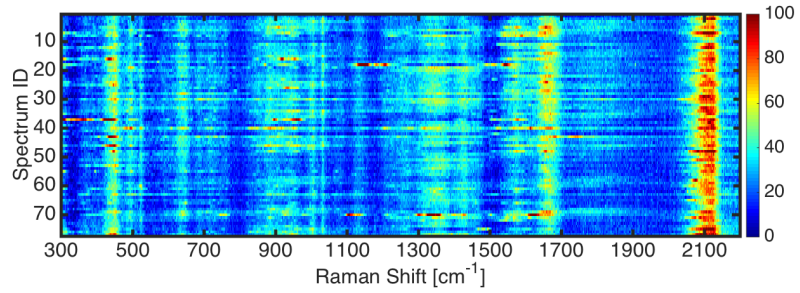
Figure 34: 1 g/l melamine in milk drop on Si-Au substrate. The top 16 spectra are from the droplet. Exposure of 3 x 1 s to 0.1 mW laser power.

Based on the droplet test melamine could be detected when the surface coverage was thin enough and laser power together with exposure time set to avoid major photobleaching and fluorescence. In practice, depending on surface deposition, laser power of 0.1 to 3 mW and exposure of 1 to 3 s were used. These values could be exceeded, however setting the focus with very low or high laser power is difficult in milk. Also working with untreated milk samples outside of this window lead to increasing amount of useless spectra with a negative shift or fluorescence.

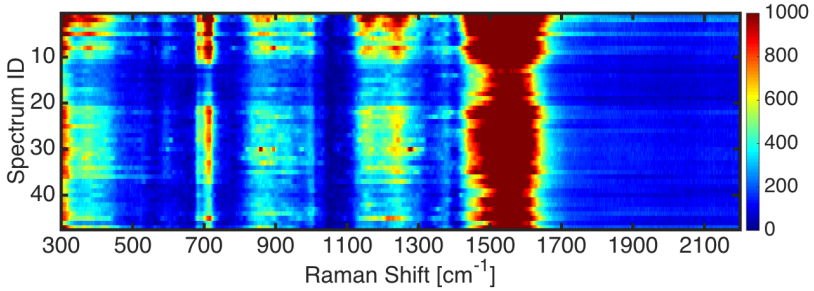
Not only is finding the characteristic peak in a very narrow area on the edge of a droplet an issue, but the droplet itself causes a statistical problem too. Since the segregation, the drying of a droplet produces a varying deposition rate of substances on the chip. As it is impossible to make identical droplets, it is nearly impossible to estimate the concentration gradient along the direction of drying in the droplet. Linking the gathered signal to concentration in such sample bares sampling error that is very difficult to evaluate [Gy, 2004b]. In addition, measuring melamine signal inside the droplet failed due to high noise and most probably decomposing of material. Thick depositions may even retain moisture.

6.3.1 Towards robust selection of sampling area

To obtain larger areas suitable for melamine detection and robustness in the selection of sampling spot, the chips were dipped into milk samples for $\sim$ 2 to 5 s. The edges of the chips retained solution on the chips that deposited even more material on the substrate. To diminish the amount of excess solution an edge of the chip was gently laid against a clean wipe that allowed milk to pour off the substrate. The result was still an almost fully covered surface, but detection was no longer as highly dependent on the sampling spot at the edge of the drop. With this method 50 mg/l of melamine was visible in most sampling spectra in the colormap 35b at $\sim$ 690 - 700 cm^{-1}.



(a) Si-Au substrate Milk background.

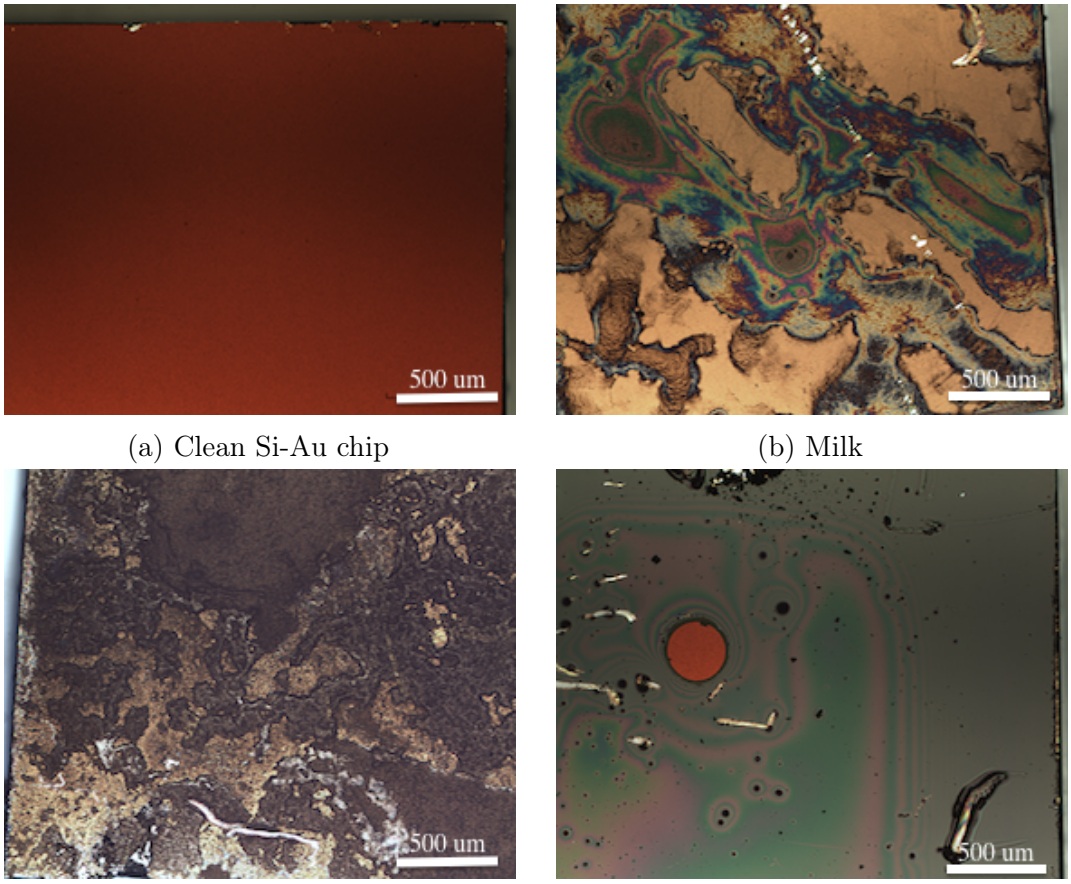


(b) 50 mg/l melamine in Milk on Si-Au substrate.

Figure 35: Substrate dipped into milk. Baseline corrected spectra.

6.3.2 SEM imaging of sample deposition

To gain better understanding about the material retained on the chips, one silicone chip dipped into sample types milk, AII and 1:1 EtOH/milk, was inspected with SEM imaging. The microscopy images of the selected chips are presented in Figure 36.



(a) Clean Si-Au chip

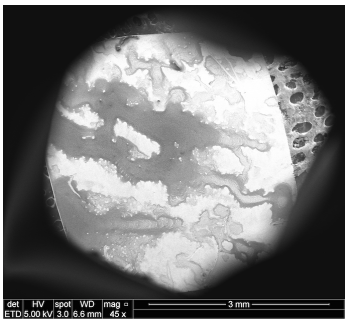
(b) Milk

(c) 1-1 EtOH-Milk

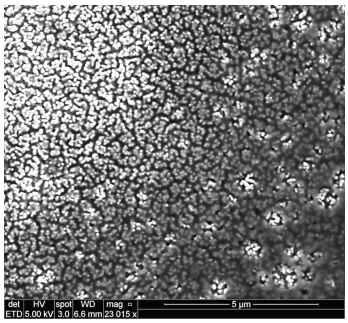
(d) AII

Figure 36: Micrographs of Au-Si SEM samples. Both milk and AII samples have interference patterns from thin deposited films. The coagula diluted in ethanol make the sample form dense clumpy accumulates on the substrate. 10X magnification.

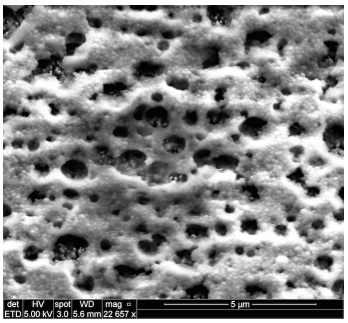
Imaging the thick depositions was difficult due to charging of the organic material. Samples of milk, Figure 37b, and AII, Figure 39c, had smoother transition zones from relatively clean to completely blocked surface. The ethanol diluted sample, in Figure 38b appeared to have denser coagulates and sharper transition to non-covered areas. The clean looking surfaces and transition zones provided suitable conditions for the SEM. The Figures 37, 38, 39 illustrate well the issues with spreading. Moreover these findings support the assumption, that surface coverage and properties of the layers on a silicone substrate have a significant impact on the detection limit. It is evident that the thick layer of coagulates do not provide good quality SERS spectra but noise from photochemistry in the organic material.



(a) Chip dipped into milk.

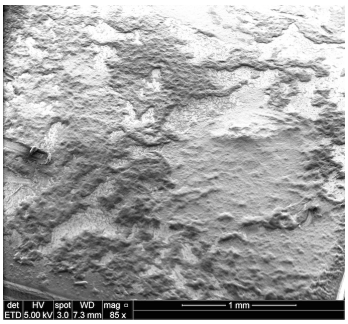


(b) Transition zone, from thin to ruptured film.

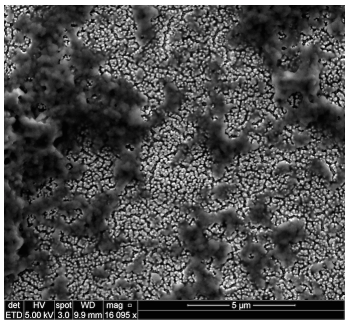


(c) Top view of a thin layer of coagula.

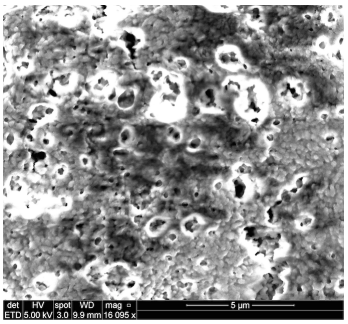
Figure 37: SEM imaging of milk covered Au silicon substrate. The areas shown bare in micrograph 36b show light in SEM images.



(a) Chip dipped into EtOH-milk. 40° tilt.

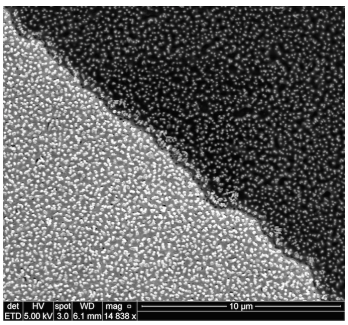


(b) Top view near coagulum.

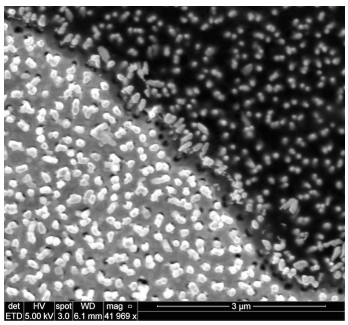


(c) Top view of a layer of coagula.

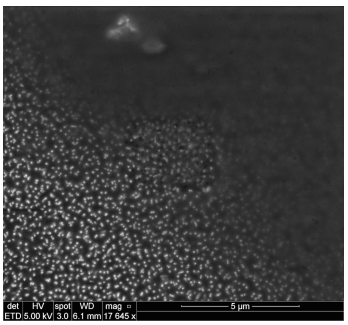
Figure 38: SEM imaging of 1-1 EtOH-Milk covered Au silicon substrate. Wide variation in deposited layers. Imaging thick layers suffered from charging, (c).



(a) An edge where an air bubble had stuck on chip.

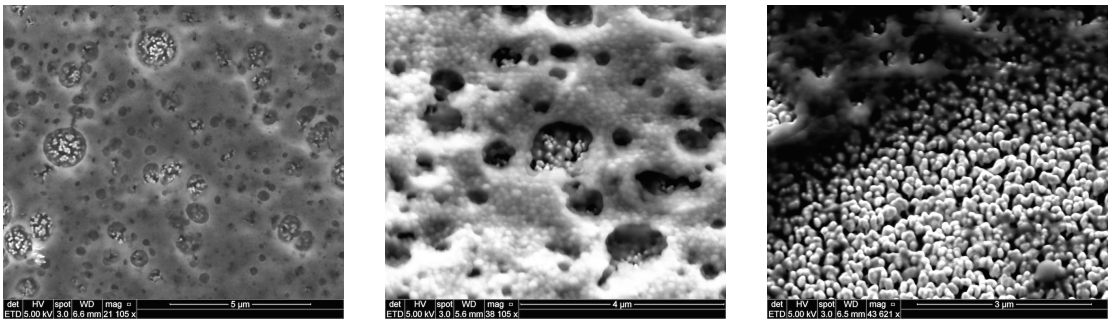


(b) 1^{st} transition zone from bare to wetted surface.



(c) $2^{n}d$ zone of the deposited film.

Figure 39: SEM imaging of AII sample on Au silicon substrate. A clear boundary layer between the light bare surface and dark blurry but uniform deposition.



(a) Milk film top view. (b) Milk film. 40° tilt (c) EtOH/Milk film. 40° tilt.

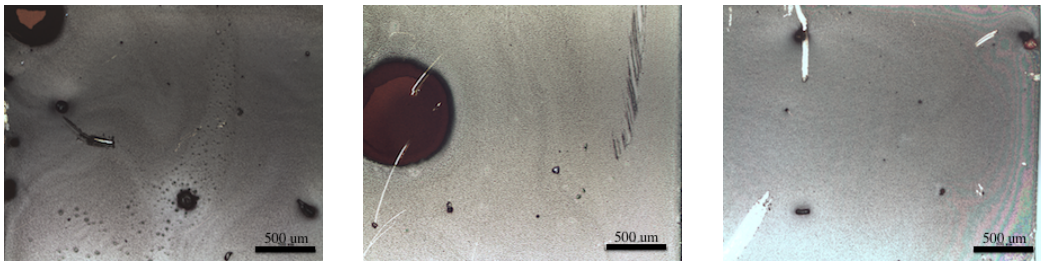
Figure 40: Deposition of Milk and 1:1 EtOH/Milk films on Au-Si nanopillars. In milk the holes are substantially smaller that the DXR laser spot size, $\sim 3\,\mu m$. The AII film did not have similar porous structure 39c.

Based on these few SEM images it would appear that the coagula indeed lies more loosely on the pillars. The body of organic compounds neither in the milk or in the 1:1 EtOH -milk samples penetrates between the grass. The porous structure of the film allows seeing the nanopillars underneath it. It is also a note worthy that the pores in the milk film are smaller than the laser spot size of $3\,\mu m$. The ratio of these "accessible" hot spots to the blocked surface could be an interesting way to approach SERS detection and the sampling problem on the substrate.

6.4 Measurement parameters optimization for milk SERS

6.4.1 Incubation time

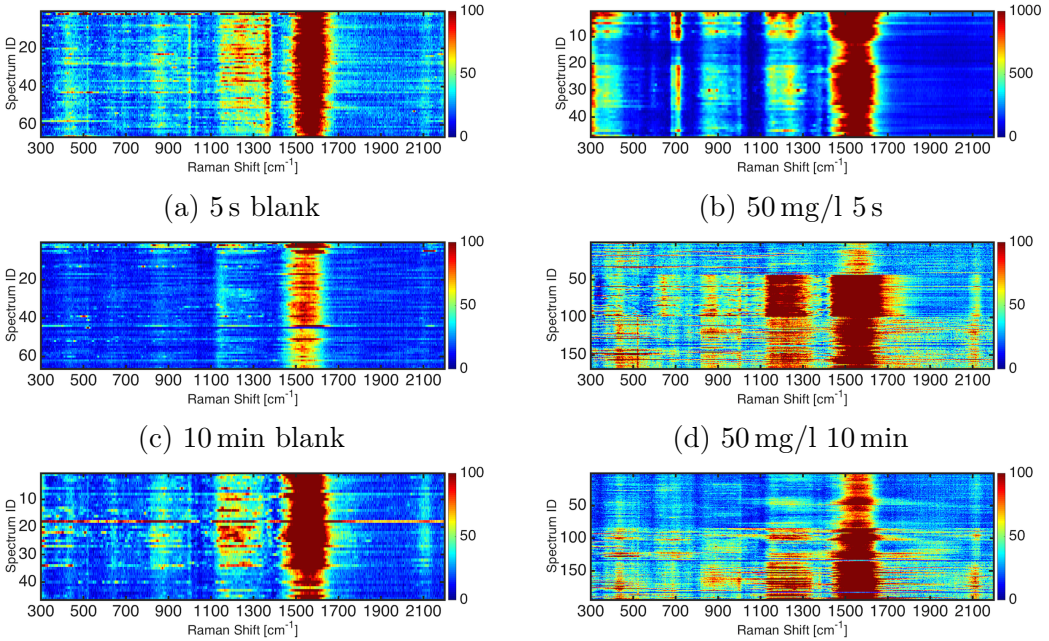
The longer contact with milk was assumed to accumulate more material on the chips which were slightly hydrophobic. Yet at apparent structure, and coverage did not change significantly between 5 s dip and 30 min immersion which is evident in Figures 41 and 50. The 1 ml solutions were kept in room temperature during the incubation. The chips from wafer *SiLT2 1* were chosen right next to each other.



(a) 5 s (b) 10 min (c) 30 min

Figure 41: Incubation test, 50 mg/l mealmine in milk. 10X magnification.

Milk did not wet the substrate entirely and small bubbles pinned on the surface. That eased focusing of the DXR as the thin boundary layer could be used for the purpose. The colormaps present the SER spectrum ranked by the ID that are the ordinal number of measurement points.



(a) 5 s blank (b) 50 mg/l 5 s

(c) 10 min blank (d) 50 mg/l 10 min

(e) 30 min blank (f) 50 mg/l 30 min

Figure 42: Incubation test SERS results, 50 mg/l melamine in milk, 1mW, 3x 3 s.

As the measurements were taken as line scans deliberately over ruptured area, the different surface coverage is visible in the colormaps. The transition between different zones is clear in Figure 42 where the higher intensity corresponds to thin and ruptured areas for example in colormap 42d the highest intensity rises from the dark wetted area inside the bubble seen in the microscopy image 41b. In the colormap 42f the measurement approached the edge of the chip where the interference pattern seen in the right bottom corner of the figure 41c.

The baseline corrected the average spectra of the milk incubation test are presented in Figure 43. The standard derivation increased with the incubation time a little but over all the results were really similar. The material was retained as quite a thick layer and lack of thin deposition in all samples ensured that on average the peak height was very modest. As major difference was not seen the simplest and least time consuming method was chosen. An additional benefit of short dipping was that chips less likely to scratch. Grabbing the corner of small chip in turbid solution possesses a risk of small accidents. The difference peak height at $\sim$700 cm^{-1} is small and further conclusions should not be drawn based on a single experiment.

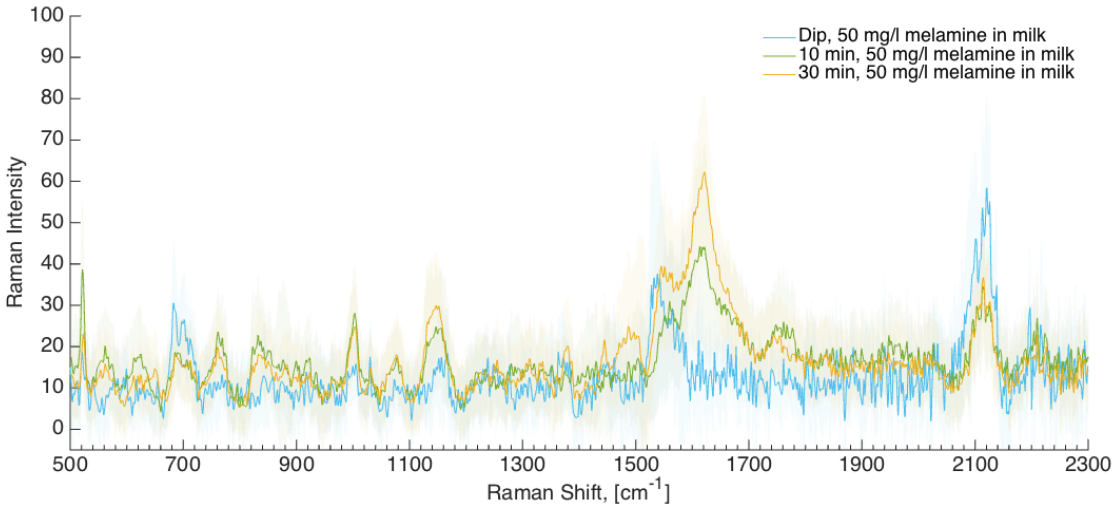
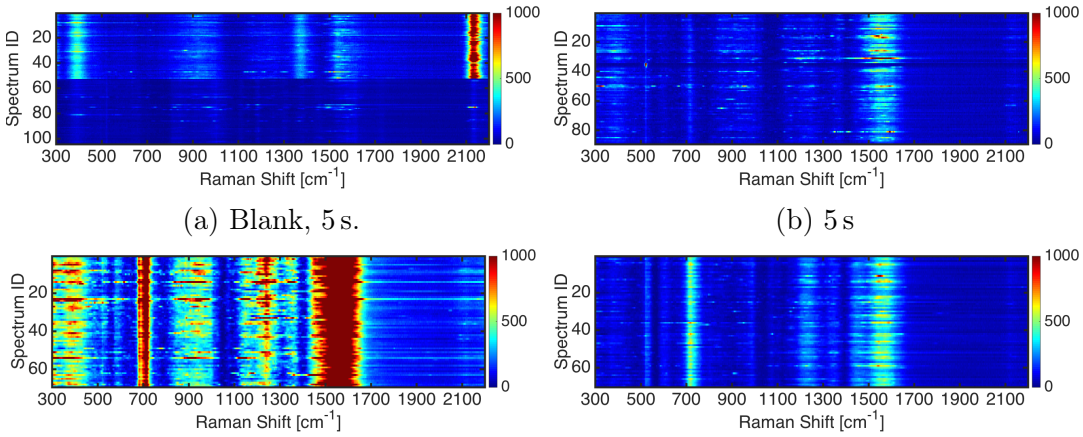


Figure 43: Incubation test in milk, 50 mg/l melamine, 1 mW, 3x 3 s. Average spectra of 60 measurement points.

Adsorption kinetics was evaluated by varying the incubation first in a 1:1 solution of EtOH/H_2O, with immersion times of 5 s, 10 min and 2 h. The best signal in 1:1 EtOH/H_2O was obtained with the 10 min immersion which can be seen in Colormap 44. The results of background and incubation in water were recorded and followed the same trend thus the data was for an unknown reason corrupted and therefore not presented here. Based on these results incubation times for milk and 1:1 EtOH/H_2O incubation times 5 s, 10 min and 30 min were chosen for ETOH/milk.



(a) Blank, 5 s. (b) 5 s

(c) 10 min (d) 2 h

Figure 44: Incubation test SERS results, 10 mg/l melamine in 1:1 EtOH/H_2O.

6.4.2 Laser power

The organic material on the chip is prone to photochemistry that makes focusing the laser really difficult on thick layers as the exposure time is long. Moreover, with a low analyte concentration focusing noisy signal without any band to look for leads into situation where the focus actually is one of the variables. To balance that out larger maps with more sampling points can be recorded as the substrate will always be tilted respect to the laser beam. The melamine band in water was successfully enhanced by increasing the laser power. Also really low 0.1 mW or high 3 mW laser power makes the operation onerous there is photoleaching together with fluorescence. When measurements were run with higher laser power than 1 mW the focusing was done first to the substrate surface with a 50X objective instead of the 10X that was used in the measurement. The laser was then set to focus with a lower laser power, 1 mW.

The response of the blank milk samples to the increasing laser power is shown in Figure 45. After the removal of the spectra with strong photoleaching and fluorescence the spectra recorded with higher power have less noise. Yet due to the above-mentioned focusing issues large part of the measurements have been taken with 1 mW laser power. In water the wider slit deduced the peak intensity as seen in Figure 46 also the double peak shape changed but no reason for the change could be addressed.

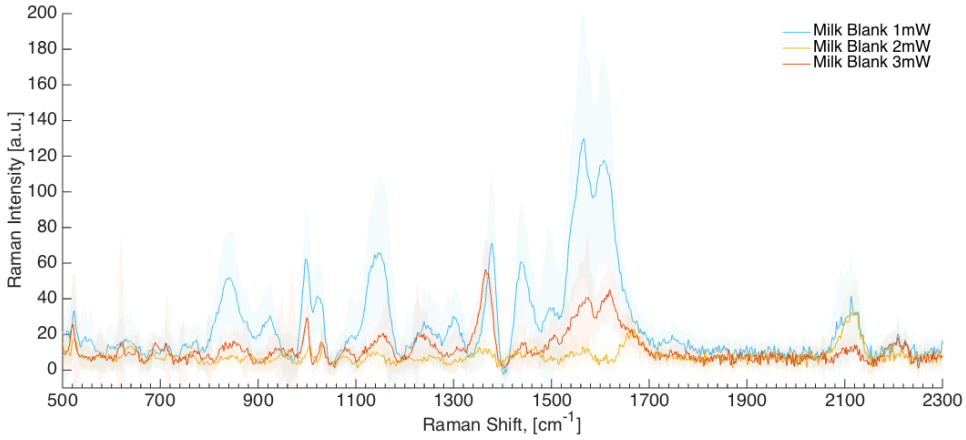


Figure 45: Background of milk, 1, 2 and 3 mW laser power, 3x 3 s. Baseline corrected average spectra of three measurements of 60 measurement points on *Si5003*.

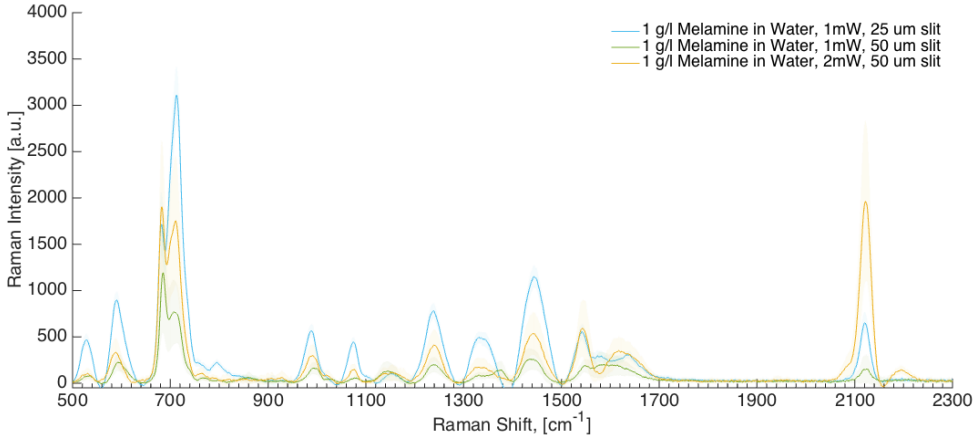


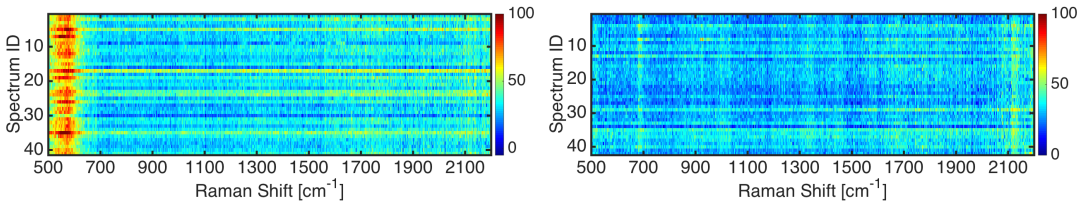
Figure 46: Change in 50 mg/l melamine spectrum in water respect to laser power and slit size, average spectra of 50 measurement points measured on the same substrate chip.

6.5 Results from the pretreatment schemes

6.5.1 Acidic precipitation

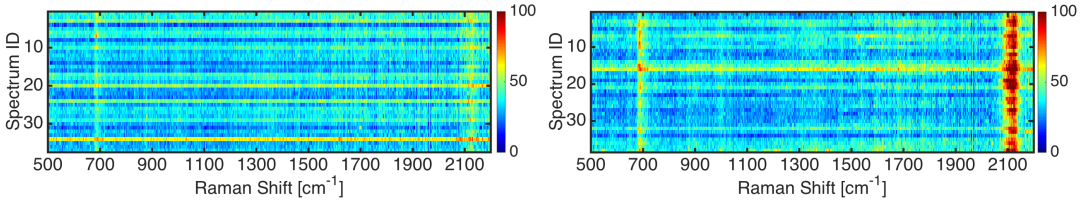
In the first set of experiments AI, AII, ACI and ACII solutions were prepared and then spiked with melamine to 1 g/l. These reflect lossless sample preparation process and so the best possible detection that could be achieved by this pretreatment. Assuming that melamine does not interfere with the separation process.

The preliminary tests showed that 10 mg/l detection was possible from a centrifuged sample, as a weak band in the colormap 49b. The potential melamine losses at this stage are solid melamine crystals, melamine cyanurate and the loss with the centrifuged fraction. The second purification step was two stage filtration as seen from Figure 48 filtration was also shown beneficial, yet it bares a risk of loosing protein bound melamine.



(a) Background. (b) 10 mg/l melamine.

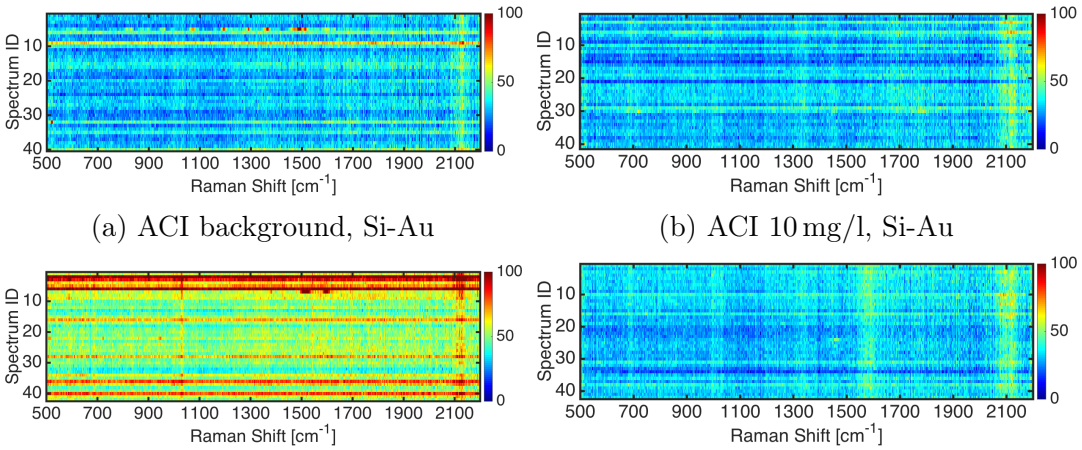
Figure 47: AI 10 mg/l- centrifuged, Si-Au substrate, 1 mW 3 x 1 s



(a) AI 50 mg/l melamine, 1 mW 3 x 1 s. (b) AII 50 mg/l melamine, 1 mW 3 x 1 s.

Figure 48: Effect of filtration, Si-Au substrate, no $CaCl_2$ added

An addition of $CaCl_2$ was expected to reduce protein originating noise but in the presence of the salt in 20 mM concentration the SERS signal was reduced and the detection limit of 10 mg/l lost. To support the assumption of salts hindering the lean or blocking the substrate by other means was tested with a measurement with 1:10 PBS-H_2O buffer solution. The intensity of peaks from the substrate background decreased to half with the buffer. The result is again fairly guide-lining and ought to be conducted with equivalent ionic strength solution similar to the milk mineral composition and presence of low concentration of melamine. In this experiment $CaCl_2$ was added after the filtration as its blocked the disk filters immediately. With other filtration method, such as cross-flow filtration or gel filtration, the addition might be useful at earlier stages when it might also prevent the possible losses of melamine associated with casein.



(a) ACI background, Si-Au

(b) ACI 10 mg/l, Si-Au

(c) ACII background, SiO_2-Au,

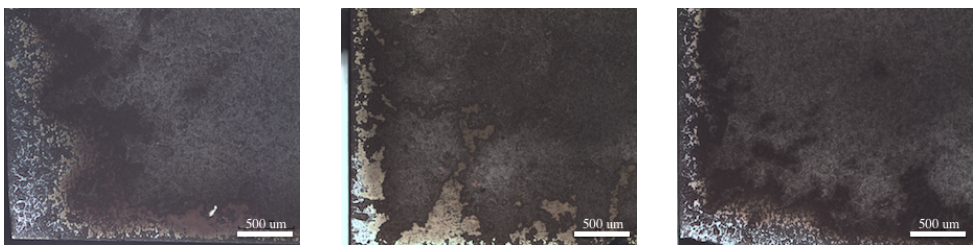
(d) ACII 10 mg/l, SiO_2-Au

Figure 49: 10 mg/l melamine in ACI sample on a silicon substrate, 1 mW 3 x 1 s and ACII sample on a glass substrate, 1 mW 2 x 1.5 s.

6.5.2 1:1 EtOH dilution

Milk was diluted 1:1 in ethanol being aware of the possible segregation of melamine in ethanol, water and organic material. The dilution was hoped to improve wetting and break the milk film to into lace-like structure with the small and evenly distributed islands of coagula. In other words, the aim was to make more heterogeneous deposition with small features that would be more even in composition and structure along the substrate on average. The low deposition will ease focusing and ethanol not only wets the surface better it also changes the way the pillars lean. A down side is the fact that trace chemical detection in general benefits from up-concentration rather than diluting.

The incubation test was carried out to EtOH diluted milk samples too. The Figure 50 shows, as expected, that milk agglomerated and the coagula formed ruptured coverage, not quite as fine as wished for, but the DXR focus was significantly better and the signal from less heavily deposited areas was well resolved. The data is not presented here because of file corruption. Based on the observations of the measurement, no high enhancement was achieved by longer incubation time.



(a) 5 s

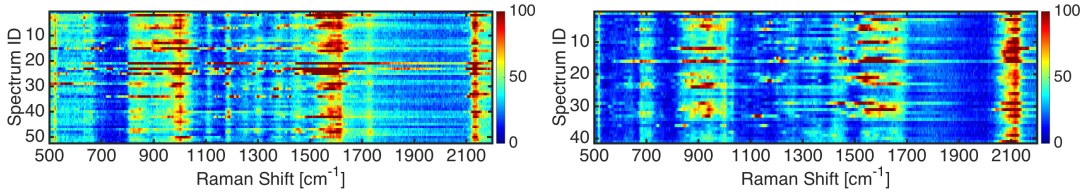
(b) 10 min

(c) 30 min

Figure 50: Incubation test, 10 mg/l mealmine in 1-1 EtOH-milk. A 10X magnification.

The assumption of gaining some enhancement in the signal with the ethanol dilution proved to be on the right track. Milk with 10 mg/l melamine was diluted 1:1 with analytical grade ethanol and chips were immersed into the solution. The results are presented in Figure 51 and the calculated average spectra in Figure 52. The $\sim$700 cm^{-1} band is less uniform along the line scan but was nevertheless enhanced. The sample was also less prone to have focusing issues even with high laser powers.

Dilution to Ethanol allowed increasing both laser power and exposure time without losing information around 600 to 1000 cm^{-1}. The sample volume of these dilutions was 1 ml. Even though sedimentation occurred the chips were immersed in the 1.5 ml tubes and excess material was run off from the chips as with untreated milk. The line scan across the deposited white material and bare looking areas showed that still the strongest melamine signals were collected near by the edges of the solid precipitates.



(a) Blank (b) 10 mg/l melamine

Figure 51: 1:1 EtOH-milk, 2 mW, Si-Au substrate.

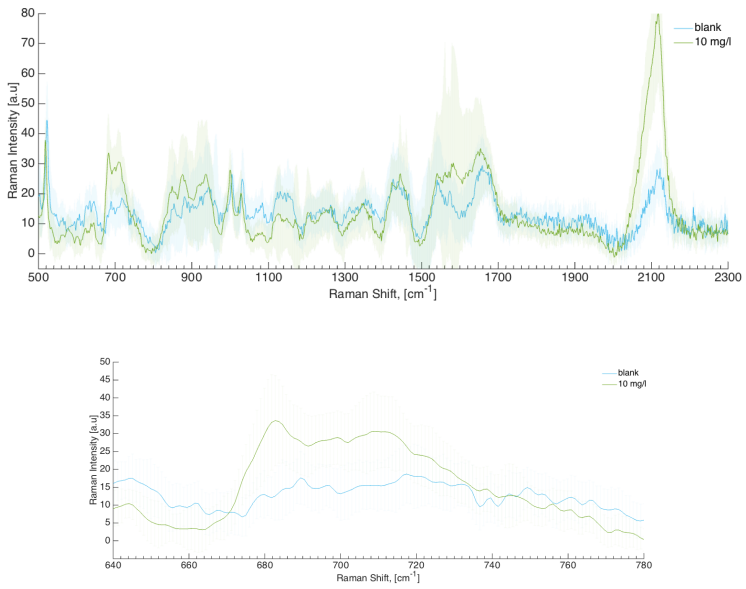


Figure 52: 1:1 EtOH-milk, 10 mg/l melamine and blank, 3x6 s, 2 mW, Si-Au substrate. The baseline corrected average spectra of results in colormaps above fig 51.

6.5.3 Addition of melamine before and after pretreatment

This experimental arrangement offers no quantitative information on melamine losses to be used for sampling statistics as the detection method itself is not validated. The aim of these measurements was to test if the SERS detection differ in two sample sets. The test could have been conducted with a dilution series however I wanted to put both the sample preparation and the SERS detection on a test.

Thereby this subsection concerns only the pretreated samples AI to ACII that have a phase separation and thereby a direct loss of melamine. There is no phase separation in pretreatment of ethanol dilution but the formation of the coagula may lead to a different distribution of melamine in solution making a real mass balance of melamine between the phases would still be interesting and relevant to the measurement accuracy. One may see that no surface enhancement happens inside the lumpy coagua.

To minimize the melamine losses in within weighing and making dilution series a larger batch of mother solution of only 50 mg/l melamine content was prepared for the experiments. The concentration was chosen based on the detection limit reached so far. Regardless the sample preparation 50 mg/l had been detectable. The solution was let to stabilize over night like the before and the following morning a dilution series of 50, 10 and 1 mg/l. The sampling was done from stirred solution where turbulence was made by immersion of a spatula to restrict the flow. The blank sample was stored and handled following the same pattern and the spiked samples did. These dilutions then went trough the sample preparation procedure. Also a untreated spiked milk sample was collected.

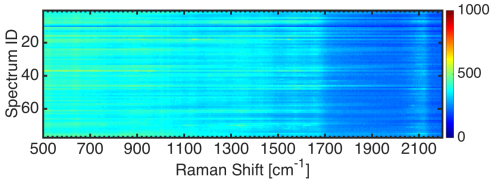
In complete contrary to the assumptions made, no melamine was detected in any of these samples. The result was also repeated by making only the AI sample and taking several experiments on the mother solution. The unsuccessful experiment revealed a possibility that melamine in low concentration can apparently react with milk and by doing so become undetectable. It is a threat to trace level detection that requires to be further examined. However, depending on the mechanism melamine hides in the solution and the kinetics. The other possibility is that it binds to storage containers in which case the storing in glass may have caused the observed issue.

6.6 The characteristic Raman spectrum of melamine in milk.

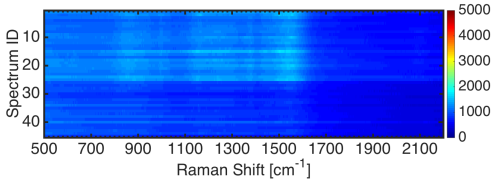
Ensemble average

The ensemble average is a powerful way to treat fundamental noise that originates from the thermally induced motions in charge carriers and is known as thermal noise. The repetitive additions of noisy signals tend to emphasize the features of systematic characteristics and, random noise is cancelled out. In milk part of the noise may origin from other compounds and the averaging method will emphasize the part too. In that sense dilution of the sample may reduce the background of the signal. The weaknesses of the method is firstly that it work better with large amounts of spectra and with high occurrence of the analyte. Secondly all the spectra within one measurement are treated the same way even if the noise was not similar as in the case of low and heavy deposition of material on the substrate. Therefore the background correction is not optimal for all the spectra. The average spectra presented in this work are sets of data smoothened with the Savitzky-Golay algorithm and polynomial baseline corrected.

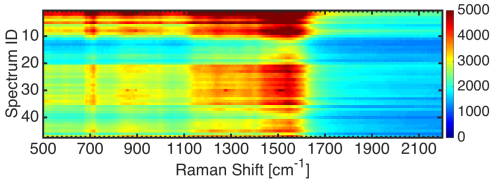
The biggest challenge in spectral analysis is the noise. Moreover, taking the averages of data that is not smoothed bares a risk of taking artifacts into the analysis. The lower the concentration more challenging the detection of the peak from the noise. Predicting the changes in peaks would require substantially larger sets of data over bigger surface area. Figure 53 shows detection of 50 mg/l melamine in water and milk, the quality of detection in these two is different. Within one line scan across a milk sample the noise may vary greatly between areas of different deposition. The variation between samples is large as seen from figures above, 54 and 55. How many sampling spots is the right amount as not all spots are equal and some in-fact are useless? The laser spot size always covers a number of hotspots but it is not known how many contribute to the signal at low concentrations.



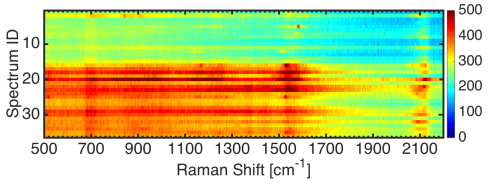
(a) Si5003 milk background



(b) SiLT2 1 milk background



(c) SiLT2 1 50 mg/l Melamine in milk



(d) Si5003 50 mg/l Melamine in milk

Figure 53: 50 mg/l Melamine in water and milk on two substraes, Si5003 and SiLT2 1, 3 x 3 s, 1 mW. Substrated dipped for 5 s

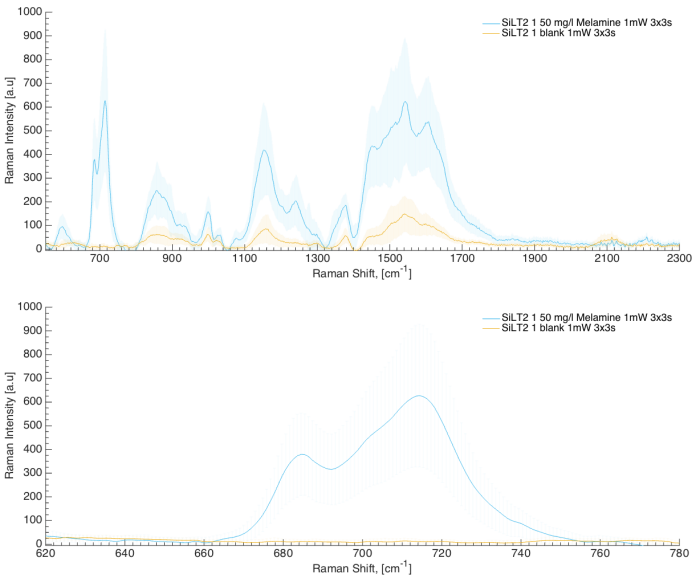


Figure 54: The spectra of blank and 50 mg/l melamine in milk. Collected on a thin deposition of milk on slightly hydrophobic *SiLT2 1* Au substrate, $3x3$ s, 1 mW.

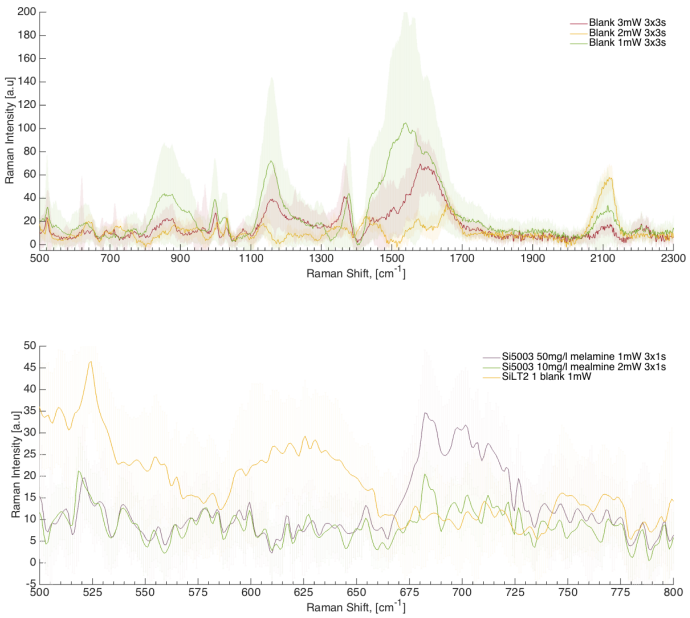


Figure 55: The spectra of blank, 10 and 50 mg/l melamine in milk. Collected on hydrophilic *Si5003* Au substrate with thick layer of milk covering the surface, $3x3$ s, 1 to 2 mW.

Comparison of the average spectra of measurement with only a thin, ruptured deposition in Figure 54 and a thick layer in 55 shows that the outcome could not arise from wafer to wafer difference in performance. In Figure 54 the thin deposition of sample gives rise to high standard deviation. Nevertheless the fingerprint peak can clearly be distinguished from the background in Figure 54. Unfortunately, the Figure 55 represents the more common case. Thick layer decreases the over all Raman intensity of the collected spectra. Increase in laser power decreased the noise, however also the line scans were taken $\sim$5 µm aside from the previous line. The ensemble average of 10 mg/l melamine in milk can not be distinguished simply by the peak hight but the shape of the spectra at $\sim$680 cm^{-1} allows detection.

The measurement in Figure 54 is one of the best detections of melamine in milk within this study. The results are from two measurement sets, of a different wafer and sample. The concentration of melamine was high enough to distinguish the characteristic band around 700 cm^{-1} at nearly every point measured making it possible to take the average spectrum of the measurement. The colormaps of the raw data of two measurements are shown below in Figure 53. On rather optimal coverage, the peak intensity exceeds values over 200 counts.

6.7 Towards trace concentration detection

The reliability of the qualitative detection rises from reproducible measurements and having statistical rules to identify the characteristic peaks. This section aims to enlighten the various challenges arising from the data.

Firstly, the peak intensity is a product of enhancement aka the adsorption and the concentration. The tests carried on by M. Schmidt, J. Hübner and A. Boisen in the figure 56 enlighten the linking between adsorption and the peak height. The leaning of the silicon nanopillars is of irreversible kind. Once the pillars lean molecules can not easily adsorb in the hotspots.

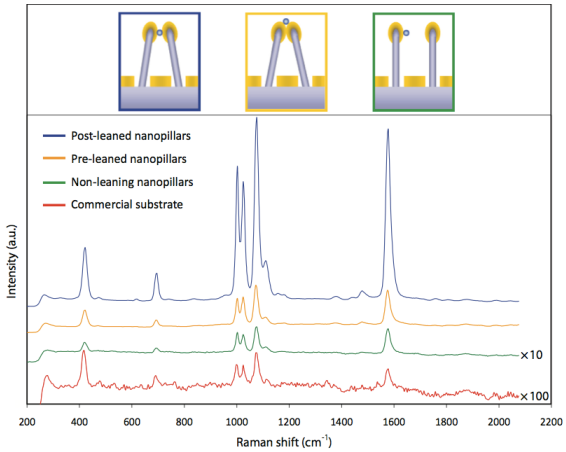


Figure 56: Adsorption site influences the signal intencity [Schmidt et al., 2012]

When the characteristic spectrum is visible in all points sampled, ensemble average is less likely to bias the observations whereas with trace concentrations the distinctive information is evidently lost. This leads to demand in having several SERS samples chips with trace level concentrations probed to be able to compare the signal intensity change effectively.[Gy, 2004a] Calibration curve done at low concentration demands great precision and large SERS mapping area. Moreover preparation of trace level solution always has high sample preparation errors in dilution series. The calibration curve prepared should comprised of several dilution series.

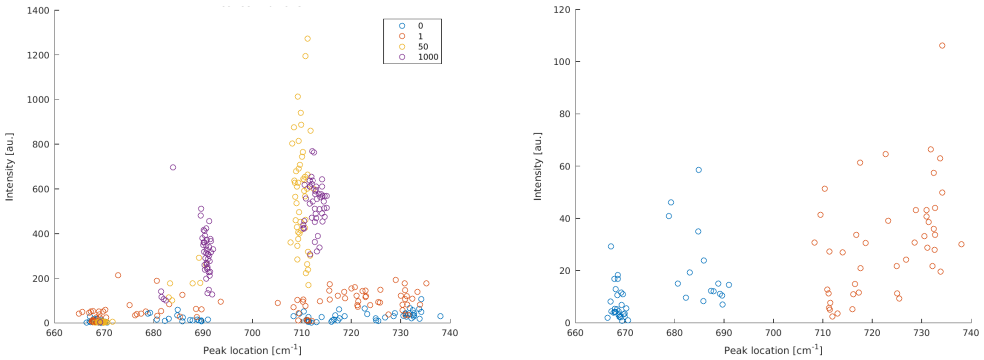
The concept of trace amount in solution is an interesting question in this context. The distribution of trace chemicals can't be estimated with normal distribution but follows more likely Poisson distribution. The detection limit of melamine in milk has been set to 1 ppm. In volume of 1 µl of this concentration there are roughly over $4 * 10^{12}$ molecules. The assumption stands only when there is no interaction between the analyte molecules. In water melamine is easily detectable and within laser spot size of approximately 3 µm there are always hot spots.

7 Analytical Methods and Data Analysis

The ensemble average suppresses random noise when the repetitive patterns in the measurement spectra are consistent. The lower the concentration more likely the spectrum of interest is turning into deviation from otherwise noisy spectra. Before further data handling, relevant spectra arising from melamine have to be extracted from the measurement data. Simply the peak height at given wavelength is no accurate enough to use as a base of the detection.

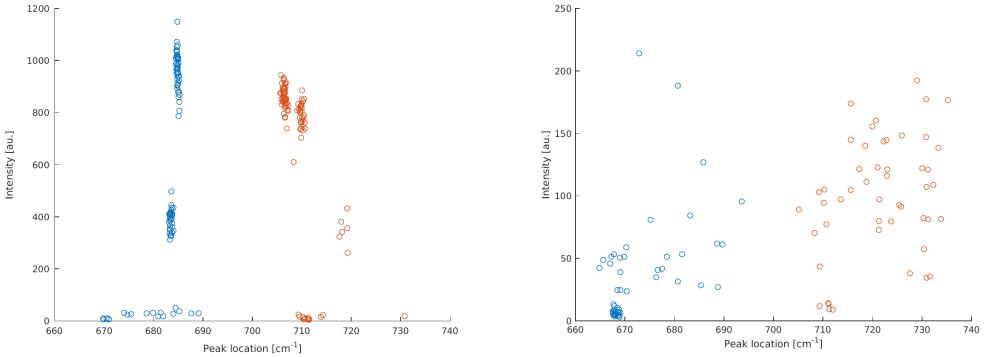
7.1 Peak Fitting

Analysing results with 1 mg/l concentrations of melamine in milk need more sophisticated statistical methods than averaging to separate weak peaks from noise. F The first step towards reliable data analysis is finding the spectra corresponding to melamine with a sufficient level of confidence. Use of algorithms that utilize Gaussian and Lorentzian peak fitting allowed plotting graphs in Figures 57 and 58. The peak collected is often a double peak but not always which is a challenge for peak recognition. It is to be pointed out that the data is only a very small set of measurements and there is no proof of reproducibility. Simply finding a peak of desired shape and sufficient height in the observed wavelength region is not sufficient for detection. As already mentioned a single peak or a double peak at $\sim 680-715\text{cm}^{-1}$ may arise from other compounds with similar structure as the melamine triazine ring. The melamine spectra have to be distinguishing from other compounds in milk and contaminants before statistical analysis.



(a) Milk all concentrations (b) Milk blank

Figure 57: Peak positions in milk samples 0, 1, 50 and 1000 mg/l melamine.



(a) Water 1 g/l (b) Water blank

Figure 58: Peak positions in H_2O

Peak Fitting Model

The results above in Figures 58 and 57 are obtained with an in-house peak fitting model developed at DTU Nanotech by Tommy Sonne Alstrøm *et al.*. The signal response model for a spectrum is

$$f(x) = p(x) + b(x) + \epsilon \tag{2}$$

where $f(x)$ is the recorded spectrum, x denotes the wavenumber, $p(x)$ is the peak model, $b(x)$ is the background model, and ϵ is the noise model. The peak model used is the pseudo-Voigt function which is a linear combination of a Lorentzian shaped curve and a Gaussian shaped curve defined as [Wertheim et al., 1974]

$$p(x,x_0,w,\eta) = \eta L(x,x_0,w) + (1-\eta)G(x,x_0,w) \tag{3}$$

where x_0 is the center location of a peak, w is the width of a peak and *eta* controls the shape of a peak. Physically, the Lorentzian shape is considered the response from the vibrational states of molecules (ref Tomas) and the Gaussian shape is the associated vibrational noise. The Lorentzian curve is

$$L(x,x_0,w) = \frac{1}{1+\frac{(x-x_0)^2}{w^2}} \tag{4}$$

and the Gaussian curve is

$$G(x,x_0,w) = \exp\left(-\frac{\ln 2}{w^2}(x-x_0)^2\right) \tag{5}$$

Under the given model, the recorded spectrum is assumed to mostly contain measurement noise besides peaks and background which makes a normal distributed likelihood appropriate

$$x \sim \mathcal{N}(Ap(x,x_0,w,\eta),\sigma_{noise}) \tag{6}$$

where A specifies the intensity of the peak at x_0. In order for Bayesian inference to be eligible prior distributions on the parameters are required.

Comparing results from different wafers and even chips from the same batch proved to be tricky. The coverage, the choice of sampling spot, focus and likely also the age of the substrate together have an impact on the appearance of the band at $\sim$700 cm^{-1}. Still the above used method indicate that 10 mg/l of melamine in untreated milk could be detected. However, it is not yet possible to state the level of confidence of the results.

7.2 Pattern recognition with multivariate analysis

The peak fitting provides a tool to find Raman shifts at the region of interest. The method evaluates the whole spectrum but very little information on the quality of the collected spectra is gained. Multivariate analysis can be used in preprocessing, feature extraction and classification of the data. The aim is to find meaningful patterns. The benefits of classifying and feature extraction is that the melamine originated spectra can be separated from the noise with systematic mathematical method. Furthermore, the data can be ranked the sampling spots compared to evaluate the effective sampling frequency.

Bayesian Non-negative Matrix Factorization (NMF) is an unsupervised learning method. It has for instance been utilized with promising results in less complex media but at lower concentrations. As an example, results from 17β -Estradiol detection application benefiting from the NMF method are shown in Figure 59 [Alstrøm et al., 2014]. Band-Target Entropy Minimization (BTEM) has also been utilized in FTIR and Raman spectral reconstruction. [Widjaja et al., 2011] & [Chew et al., 2002] The method also targets the reconstruction of individual pure component spectra. In Figure 59 the colormap presents the intensities of a specific Raman shift where the target molecule has a peak too. The peak height at the chosen wavelength may arise from fluorescence and impurities too. Local contaminants (green and red lines) and analyte originating spectra (blue line) can be separated with pattern recognition.

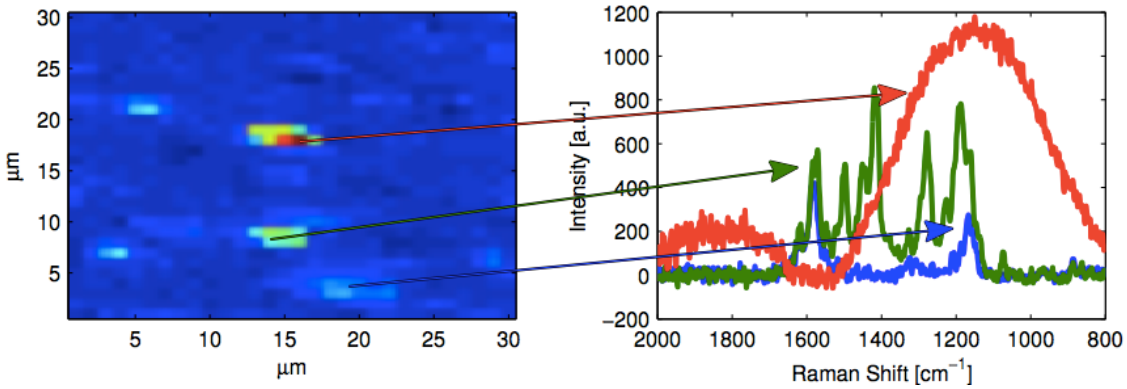


Figure 59: An example pattern recognition with Bayesian non-negative matrix factorization. The Raman map (left) has local contaminants and spectra (right) originating from analyte (blue line) and impurities (red and green) [Alstrøm et al., 2014]

The NMF method was applied to treat the measurement data in Figure 60. The spectra from measurement 50 mg/l melamine in milk were surged for five patterns and the results are presented in Figure 61. The map a) shows the line scan run over the chip and the colorscale correlates to how strongly the pattern in b) is present in the spectrum collected at the point.

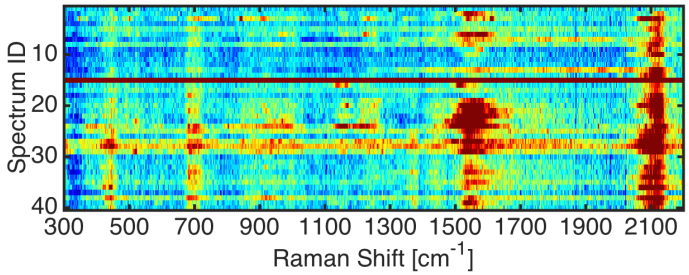


Figure 60: The baseline corrected data used for testing Bayesian NMF. 50 mg/l melamine in milk, Si-Au substrate.

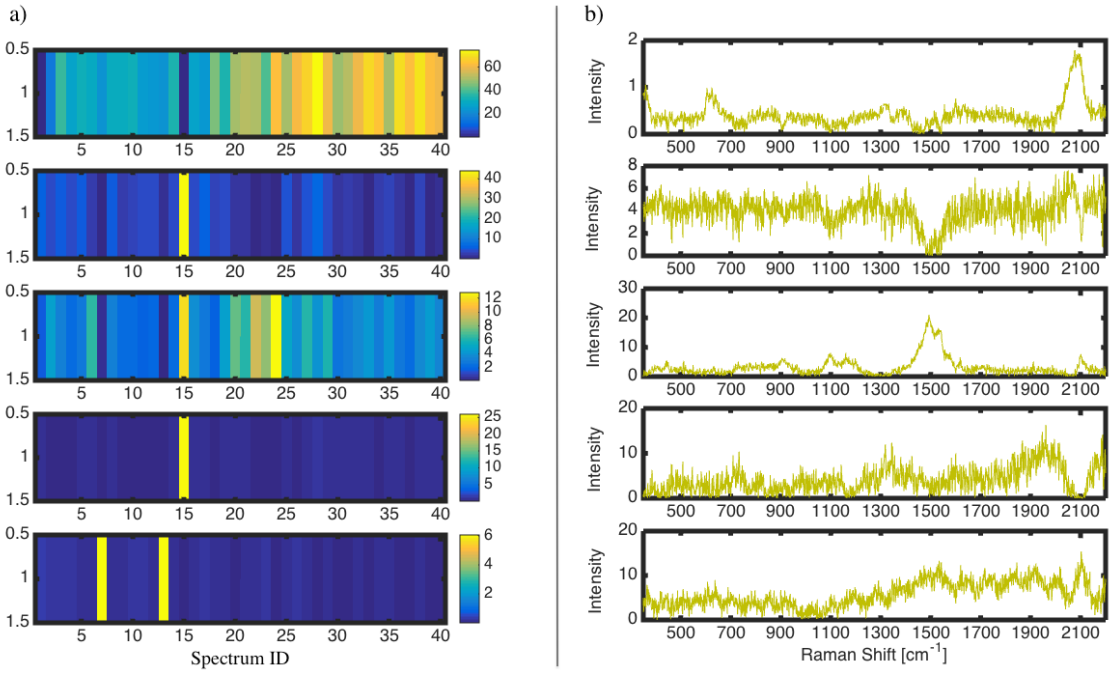


Figure 61: An example Bayesian NMF on a milk sample with 50 mg/l melamine, Si-Au substrate. a) The location of the found pattern in the corresponding Raman map. b) The found five patterns.

The method found the strongest pattern with Raman shift at $\sim 615\,\mathrm{cm}^{-1}$. The band in the original data however appears to be be higher, at $700\,\mathrm{cm}^{-1}$. The difference could arise from baseline correction or fault in data pretreatment. Nevertheless the pattern recognition used within this work is still work in progress and the concept is promising.

8 Heterogeneous Mixtures - Practice of Sampling

8.1 Sampling from milk

This section is about how one should select a sample and how much material should be selected. As there is no such thing as a constant sampling bias as sampling is mass reduction and highly dependent on the manner each reduction step in done. For the present purpose, we can summarize that:

$$Qualityestimation = Sampling + Analysis$$

In this work the Lot to be sampled is 1 l. However the suitability of the analysis should be evaluated for much larger volumes. Then the size reduction to $\sim 2\,\mu l$ is no longer a simple task. During the size reduction of milk samples, one has to keep in mind that milk has a tendency to form layers of different compositions. Thereby sampling should always be made from stirred solution, preferably from turbulent flow. Mixtures that have components of different densities and phases are driven to sediment and segregate. Also the distribution of analyte in the mixture may vary greatly. Sampling in above mentioned conditions is a difficult task. Therefore sampling in all steps from making a dilution series to the deposition of the material on the substrate must follow the same procedure in detail. This study uses UHT milk that is, true solution, emulsion and colloidal solution all at once. All sampling in should be conducted from stirred solution in turbulent flow. In practice the mixing was enhanced by immersion of a spatula near the sampling spot. Fortunately, the homogenization process decreases sedimentation and phase separation allowing 4 days of storage of milk in fridge.

According to Pierre Gy, the primary sampling biases may be as large as 1000% relatively and secondary sampling biases up to 50%, whereas analytical biases do not usually exceed 0.1 to 1% [Gy, 2004a]. In the case of the milk sample on SERS substrate the bias of analytics is poorly known. The substrate enhances only those molecules that are in the hotspots and the evaluation of distribution of molecules on the substrate is not possible from the data gathered. Therefore the evaluation of analytical error is left out of the scope of this study.

The drying of the sample on the SERS substrate bares one of the largest and least controllable errors in the sampling chain, segregation. Evaluation of distribution of melamine within milk is questionable as droplet composition and drying conditions together create a concentration gradient on the substrate. When the droplet dries, the contact line moves with varying velocity and the compounds with different vapour pressure and adhesion on the substrate either concentrate or dilute.

Segregation may also happen between solid phases: the coagulum and the substrate surface. Observing how a droplet with 1 g/l melamine dries the crystallization can be seen clearly and the forming particles travel in the droplet until attached most

often to other melamine particles forming fine structures. As the SERS measurements are taken in dry state the substrate surface is likely to saturate at a concentration where the excess melamine forms larger crystals less likely adding more molecules in hotspots. Measurements with water showed that the signal collected directly at large crystals is lower than from bare looking surface. Also the melamine bulk signal may indicate to excess amount of molecules on the substrate but it may arise from adsorption site too. The wetting of the substrate in this concept is important as the drying always directly influences the local deposition of analyte.

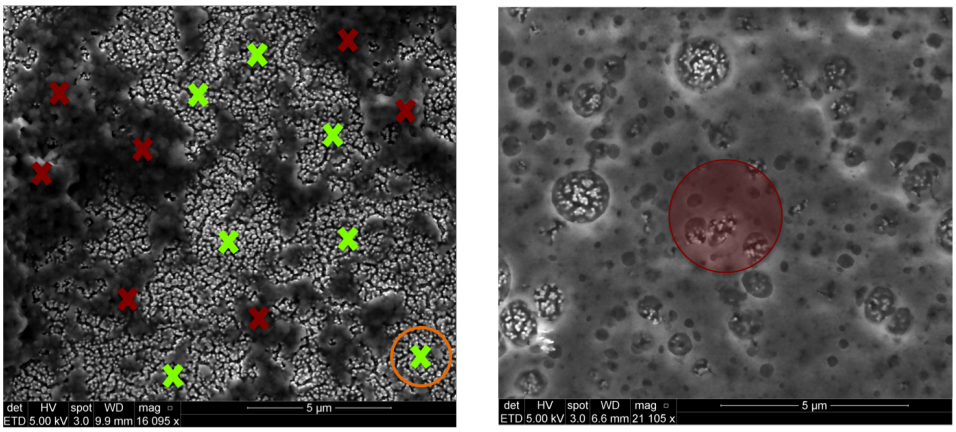
Melamine may bind to proteins in milk so creating a competitive adsorption site. Also physisorbtion on gold is possible however there apparently is no strong attraction. During this work, I found no studies on the kinetics of two sorption mechanisms and whether the protein bound melamine can be detected by SERS. The following ought to be studied to estimate the quality of the sampling chain.

- Milk sample size reduction and sufficient sample volume.
- Segregation of melamine when solution dries on the substrate.
- Study on the analytical error of the substrate with the analyte of interest.

8.2 Number of points in the sampling space

The volume of milk is reduced to a sample of some micro litres that is then dried on a substrate. The milk sample on a SERS substrate is a thin film probed with a laser. One fundamental question is how does the sampling spot on the substrate represent the volume of milk. Drying concentrates material on the substrate therefore each spot sampled with a laser represents a volume of solvent with the analyte in it. As the drying is never the same in each part of the chips, theoretically, the sampled spots are not presenting similar volume. Moreover, the choice of the sampled area of the chip affects the quality of the data. According to sampling theories of P.Gy the sampling spots should be collected with random systematic sampling strategy [Gy, 2004a], [Gy, 2004b]. That is the only way to make the link between the volume with melamine to thin (2D) film with melamine.

Silicon and fused silica substrates' wetting is different which way arise from the structure density. If the wetting of the substrate changes, the comparison of the results is not reliable. The number of molecules in the hotspots as mentioned before depends on the drying. Drying may concentrate molecules in some areas diluting others or favour cathodic or anodic areas for instance. The glass substrate appeared to promote the spreading of milk on the tips of the grass where as the silicone substrate let the solution go between the pillars. Under these different conditions, the probability of trapping the analyte in a hotspot might be rather different.



(a) Samples spots (b) The laser spot size of 3 µm.

Figure 62: Accessible or blocked surface? (a) Melamine found only in the spot with orange ring, hypothetically. (b) What is the tolerance for the surface coverage per laser spot size?

The signal is collected from an area that has several hotspots. Nevertheless, the coverage of material on the substrate varies as seen in Figure 62. Not all sampled spots are equal in SERS performance especially with ethanol diluted samples the coverage varies from bare to fully covered. Under such conditions, stating the correct amount of sampling spots becomes tricky. One way to evaluate if the sampling spot is acceptable, would be to use another chemical in higher concentration in the sample.

Addition of a molecule that does not disturb the melamine detection but would spread on the substrate the same way, could provide a mean to benchmark the sampling spot.

Chance in finding a sufficient number of hot spots with melamine per measurement point has everything to do with the spreading on the substrate. The SEM images showed that the substrate surface can be blocked by deposited layers. It can still be assumed that in concentrations higher than 50 mg/l at every measurement point there is a chance to have a hot spot with melamine, but it might not be detectable due to noise for instance. Furthermore, the intensity of the Raman band is related to enhancement by a factor and resolving that from the measured data of a blind sample is very problematic. The existence of the bulk band in spectra from low concentrations may stand on the way to quantitative detection. Uncertainty arises from sampling errors, unknown analytical error and high sampling variance together with large amount of uncontrolled variables that reduce the efficiency of statistical tests. [Gy, 2004a]

The method is far to be used as a quantitative detection method, but has potential for qualitative detection. The right amount of sampling spots has to be calculated based on the confidence level set for the detection. Without knowledge on analytical error, stating a number of sampling spots can not be done accurately. Also the number of sampling spots is linked to assumed hotspot density in the substrate and surface area available for detecting melamine. If the coverage is too high for detection, sampling the only corner with a low coverage of the chip will not make the detection any better. Better reproducibility of similar deposition was gained with sample pretreatments. The acidic sedimentation method produces very uniform coverage, where as ethanol dilution agglomerates milk creating both a rather bare surface and blocked areas. Whichever is better for analysing. Also reducing the operator fingerprint and to gain thinner films the milk could be spin-coated on the chips.

9 Discussion and Recommendations for Further Research

The variation of film thickness and spreading within milk samples deposited on SERS chips is likely too high and would over rule statistical testing. For comparable deposition, the introduction of the sample has to be automatized and only chips of the same performance should be used, even if the arraignments would lead to the loss of the best detection. Reliable statistical analysis on the detection is more valuable than obtaining the best individual spectra in a map of data points. One way to watch over the spreading could be an addition of label chemical of similar characteristics than melamine but in higher concentration. The spectra of this label should not overlap with melamine so it could be used as reference for accumulation on the substrate.

Deposition, initial melamine concentration, substrate age and wetting properties together with sample age form a group of variables that are worth investigating from the sample preparation point of view. The factorial testing of the sampling chain and the detection will evidently require a lot of work. The original plan was to make melamine mass balance in milk fractions to tackle the segregation. It is in my opinion essential to be able to evaluate accuracy with a quantitative, preferable mass based, method. Otherwise the pretreatment procedures add an uncontrollable sampling error to the detection process. The reason for having to let go of this part was simply lack of time.

Knowing the melamine distribution within milk or in the pretreated sample one could build a model of concentration of detectable melamine in the solution. The adsorption of melamine is the key factor in detection. Furthermore the substrate being a new invention there are no sufficient data on the electrochemical characteristics of the surface in water solutions. Milk is highly saturated with electrolytes and the large surface area is readily adsorbing contaminants. Keeping an open mind and exploring different measurement methods to my opinion is important. The drying causes concentration gradient and segregation but likely influences the leaning as well, as seen in the SEM images where the pillars at the droplet edges lean differently.

Wet state possesses both challenges and changes of new kinds of detection opportunities with the transparent substrates. Exploration of performance under for instance different flow regimes if successful the application would be one step closer to on-line detection.

One of the biggest questions that remain unsolved is about what would be the statistical method to ensure reliable detection. Variation from wafer-to-wafer, chip-to-chip and between sampling points with various depositions of material requires careful evaluation. As with melamine the detection limit is not in fact truly trace concentration detection, but an inverse problem of the same kind as speech recognition rising simply rising from the difficult media. That is why increasing the amount

of measurement points is not necessarily the most efficient way to improve detection. Surely, higher amount of hot spots being probed returns higher chance in finding a molecule with the best possible enhancement. Linking the result to a concentration is still going to a challenging task and the perfect enhancement may turn into false positive if too much weight on single observation is given. Even though the estimation on the analytical errors of SERS was not part of this work it can not be completely ignored. The quality of the data produced and the analytical error are inseparable as the latter sets the requirements on the nature of the data and so further on to milk sample pretreatment.

Moving towards the detection of real trace concentrations will likely benefit from up-concentration such as gel filtration. For industrial sampling the sample size should be drastically increased not only for the sake of statistical reliability but also to gain confidence in the potential user. Small collective samples may be difficult to take from processes and require more sample fractioning. That is notoriously often done neglecting proper sampling strategies.

Within this project, the sampling areas were chosen anything but randomly to gain understanding about the detection from different looking areas. Linking the meta-data to spectra is easier said than done as the instrumentation does not promote the collection of the measurement related information in high quantities. Yet, the characteristics of spectra form different deposition layers could help develop more sensitive algorithms for detection.

As the experimental work done rose in fact more questions than provided answers. The SERS detection with gold coated nanopillar substrate has potential as field device sensor for melamine-like compounds in milk at least in relatively high concentrations, $\geq 10\,\mathrm{mg/l}$. Nevertheless a validation of the detection is needed. The target detection limit of $1\,\mathrm{mg/l}$ melamine in milk was achieved in one measurement with the help of advantaged data handling from Tommy Sonne Alstrøm. The work on spectral analysis could be continued with the implementation of pattern recognition algorithm Band-Target Entropy Minimization (BTEM) that has already been used in Raman spectrum analysis [Widjaja et al., 2011] & [European Commission Press Release Database, 2012].

The technology introduced in this thesis work has still many requirements meet on the way to a viable product. Measurements taken were on one single milk product type and good performance in other milk types is vital. The structure of milk varies greatly within dairy products. Alongside of the analytical challenges there are questions to answers on the substrate lifetime and storage. The designing of the instrumentation, that has naturally not been even started, should in the future focus on meeting the conditions of sustainability too. Development of analyser for less saturated media as natural waters is likely an easier task. The target for the melamine detection in milk should be a qualitative analyser with low cost, robustness towards milk composition and reliable, simple operation.

To overcome the previously discussed issues following improvements are strongly encouraged.

- Study of melamine distribution in milk and segregation - Mass balance on melamine in phases of milk.
- Optimizing milk spreading on the substrates - Spin coating.

10 Conclusions

The experimental plan laid in February was naturally rewritten several times as the experiments were more about finding a protocol, parameters and evaluation of the sampling process. Yet despite the large amount of data gathered, only little statistical testing could have been done. The reasons for lacking tools for testing the data arise from too many variables that influence the collected spectra. Bayesian analysis could provide suitable means to obtain information on the relevance of the variables but first the peak recognition from the noise should be more reliable. Linking the peak intensity to low concentrations suffers from the poor peak recognition and reproducibility of the experiments.

The milk samples had poorly controllable spreading behaviour on the substrates. The amount of retained material on the substrate mainly determined the noisiness of the spectra. A set of measurements was conducted were 60 measurement points were selected from high and low areas of substrate chips from three different wafers. The preliminary findings showed that relatively low laser power, 1 mW laser power and 1 s exposure the noise from the highly covered areas was lower than from the areas were the coverage was low. The background noise increases when the coverage is lower. The reason may be that high coverage decreases the enhancement as respectively smaller part of the sample is in the hotspots and the silicon pillars lean weaker. Furthermore, statistically the detection of melamine will suffer when the coverage is thick if the analyte is dispersed evenly in the drying milk. Under such conditions, smaller amount of molecules is detectable in the hotspots. However, no adsorption of melamine on a thin film of gold nor the association of the molecule with different phases of milk is investigated in this work.

The handling of the measurement data indeed is difficult and during this work I found no usable models for the analytical error in milk samples on the SERS substrate. Currently there are on viable means to distinguish the peak height being either product of enhancement or concentration or combination of both. This rather fundamental property that allows the detection of ultra low concentrations also bares a statistical problem especially in complex media that stands on the way to quantitative detection. The estimation of the quality of the recorded data on the other hand is an easier task. Pretreatment of milk improves the quality of the spectral data one way or another. The ethanol dilution increases differences between sampling spots and makes it easier to search for signals that are strongly enhanced. The acidic sedimentation and filtration again homogenized the data and made the melamine fingerprint band visible in nearly all measurement points at the concentration of minimum 10 mg/l when the dilution is made from high initial concentration.

Pretreatment or not, comparing results from different wafers and even chips from the same batch proved that the substrates are very sensitive and their performance varies. The methods used within this work do not yet reach a satisfying level of reproducibility. As many variables have an impact on the appearance of the band at $\sim$700 cm^{-1}.

As a starting point to future research two issues should be looked into. Firstly, standardizing and automation of introduction of sample solution to on the substrate and Secondly gaining a better understanding about the aging of the chips. The data processing challenges and reproducibility of the measurements are inseparable and the best spectra for processing the batches of data should be taken as the target for sample preparation. Obtaining single well resolved spectrum in data array has little value if the confidence level is not stated.

To conclude there is a demand for new affordable qualitative milk analytics. Milk is a high volume industrial product with vast quality variance around the world. Better knowledge on segregation within the phases and on the substrate might also have future value when developing SERS detection for other molecules of interest in milk.

- Control over substrate aging - Dynamic contact angle measurements.
- Benchmarking solution in the milk samples to monitor the spreading and the substrate performance.
- Up-concentration with gel filtration.
- Use of pattern recognition techniques (NMF or BTEM) in data treatment.

After all there is a demand for new affordable qualitative milk analytics. Milk is a high volume industrial product with vast quality variance around the world. Better knowledge on segregation within the phases and on the substrate might also have future value when developing SERS detection for other molecules of interest in milk.

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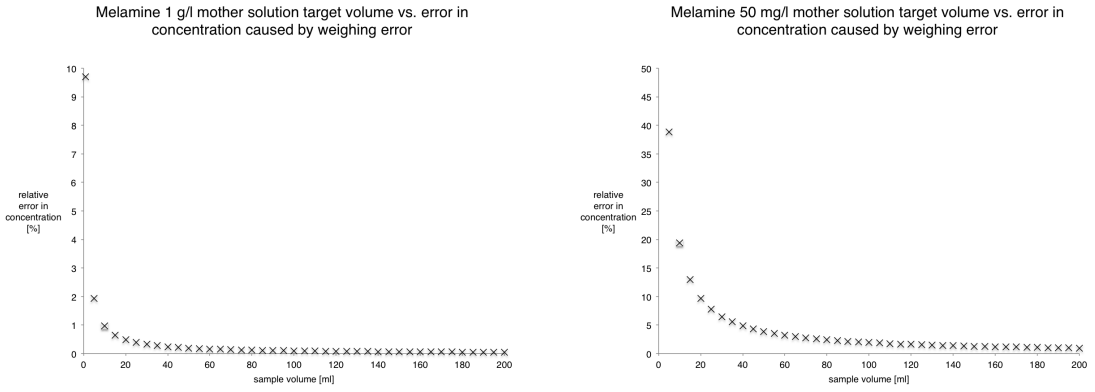
A Annex

Chemicals:

Melamine - Sigma-Aldrich Melamine 99% M2659-5G, LOT# MKBN7698V, PCode 1001644475, CAS: 108-78-1
BPE = 1,2-di(4-pyridyl)etylene, assay 97 % CAS: 13362-78-2.
Milk - UHT milk (1,5% fat, Arla) Cyanuric Acid, CAS: 108-80-5, Sigma-Aldrich pCode 185809.
Ammelide, 645-93-2, Fluka
EtOH, ACS reagent, Sigma-Aldrich

Stock solutions' weighing error

Relative error in stock solutions' concentration caused by the weighing error:



(a) Weighing error on 1 g/l stock solution

(b) Weighing error on 50 mg/l stock solution

Figure 63: Error in melamine stock solution concentration caused by the weighing error vs. stock solution target volume.

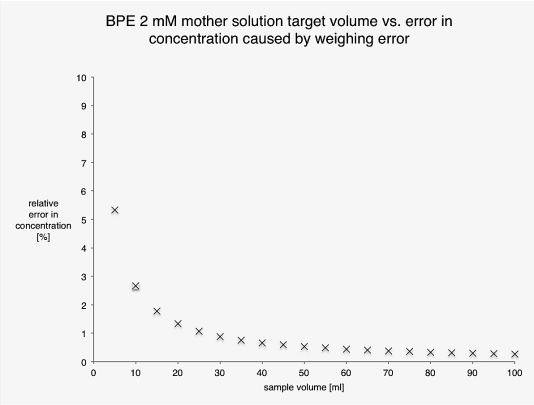


Figure 64: Relative weighing error 2 mM BPE solution.

Instrumentation:

- SERS: DXR Raman Microscope, ThermoScientific with 780 nm laser Optical Microscope: Zeiss Axio Scope.A1.
- ESEM Quanta FEG
- Dicing: Laser Micromachining Tool microSTRUCT vario, 3D-Micromac AG.
- pH meter: VWR pH110 with VWR thermometer and electrode (European Catalogue No.662-1785)
- Precision scale ACCULAB ALC 110.4 +-0.1 mg
- Advanced Silicon Etcher, ASE (STS MESC Multiplex ICP serial no. 30343).
- Advanced Oxide Etcher, AOE (STS MESC Multiplex ICP serial no. 32843).
- Alcatel SCM 600 E-beam and sputtering deposition system.
- Wordentec QCL 800
- Rapid thermal annealer (RTP) from Jipelec
- Hotplate: FTIR600 Temperature Controlled IR-stage from LINKAM Scientific Instruments